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Biotechnological prospects of rhamnolipids: production, surface tension activity, environmental applications and antimicrobial therapeutic potential

Ana Maria Martinez Torres
Magister Scientiae

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Dissertation submitted to the Agricultural Microbiology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

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Para las personas maravillosas que, con su amor,
enseñanza y guía, han moldeado mi vida.

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To my parents, Fabiola and Gustavo, whose love bridges every distance and has carried me farther than any map could show. I may have never found a four-leaf clover but having you both has always been my greatest luck.

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“If I have seen further, it is by standing on the shoulders of giants.”
(Isaac Newton)

ABSTRACT

TORRES, Ana Maria Martinez, M.Sc., Universidade Federal de Viçosa, January, 2026. **Biotechnological prospects of rhamnolipids: production, surface tension activity, environmental applications and antimicrobial therapeutic potential**. Adviser: Marcos Rogerio Totola.

The assessment of rhamnolipids as multifunctional biosurfactants has become increasingly relevant due to their expanding roles in both environmental protection and healthcare applications. In this context, the main objectives of this dissertation were: (i) to provide a detailed and comprehensive review of rhamnolipids and (ii) to generate new knowledge that deepens our understanding of rhamnolipid–environment interactions, particularly regarding their antimicrobial potential and environmental applications.

This dissertation is composed of two complementary chapters. The first chapter presents a comprehensive review of rhamnolipids, addressing their biosynthetic pathways, ecological and physiological functions, and physicochemical properties. Additionally, this chapter examines their potential as environmentally friendly alternatives to synthetic surfactants and as tools in the biomedical field, including their reported anti-cancer, antitumor, and antimicrobial activities. The chapter also discusses the main challenges that currently limit the large-scale application of rhamnolipids, such as high production costs, variability in biological performance, and biosafety concerns associated with pathogenic Pseudomonas strains.

Chapter II focuses on the characterization of three Pseudomonas aeruginosa strains from the culture collection of the Laboratório de Biotecnologia e Biodiversidade para o Meio Ambiente (P. aeruginosa LBBMA 58, LBBMA 79, and LBBMA 92). These strains were identified by partial sequencing of the taxonomic marker genes rpoD, gyrB, and 16S rRNA. Additionally, rhamnolipid production by each strain was quantified, and their congener composition was subsequently determined. This chapter includes a physicochemical assessment of the rhamnolipids produced by the three P. aeruginosa strains, including their potential as emulsifiers and surface tension–reducing agents. Their biological activities were also evaluated, considering both environmental applications and antimicrobial potential. The bacterial species Escherichia coli and Staphylococcus aureus, as well as the fungus Corynespora cassiicola, a phytopathogen of importance in soybean crops, were used as model microorganisms.

Keywords: biosurfactants; characterization; antimicrobial agent; interactions; biosafety

RESUMO

TORRES, Ana Maria Martinez, M.Sc., Universidade Federal de Viçosa, janeiro de 2026. **Perspectivas biotecnológicas dos ramnolipídeos: produção, atividade de redução da tensão superficial, aplicações ambientais e potencial terapêutico antimicrobiano**. Orientador: Marcos Rogerio Totola.

A avaliação dos ramnolipídeos como biossurfactantes multifuncionais tem-se tornado cada vez mais relevante devido ao seu papel crescente tanto na proteção ambiental quanto em aplicações na área da saúde. Neste contexto, os principais objetivos desta dissertação foram: i) fornecer uma revisão detalhada e abrangente sobre os ramnolipídeos e ii) fornecer novos conhecimentos que aprofundem a nossa compreensão das interações ramnolipídeos-ambiente, incluindo sua ação como agentes antimicrobianos e sua aplicação no campo ambiental. Tendo em conta isso, esta dissertação é composta por dois capítulos complementares. O primeiro capítulo é composto de uma revisão abrangente centrada unicamente nos ramnolipídeos, abordando as suas vias biossintéticas, as suas funções ecológicas e fisiológicas, bem como as suas propriedades físico-químicas. Adicionalmente, esta dissertação visa investigar o seu potencial como alternativas ecológicas aos surfactantes sintéticos e como uma ferramenta no campo biomédico, atuando como agente anticancerígeno, antitumoral e antimicrobiano. Ademais, este capítulo explora os desafios que atualmente limitam a aplicação em larga escala dos ramnolipídeos, incluindo os elevados custos de produção, a variabilidade no desempenho biológico e as preocupações de biossegurança associadas a estirpes patogénicas de Pseudomonas.

O Capítulo II centra-se na caracterização das três estirpes de Pseudomonas aeruginosa pertencente à coleção de culturas do Laboratório de Biotecnologia e Biodiversidade para o Meio Ambiente (P. aeruginosa LBBMA 58, LBBMA 79 e LBBMA 92). Estas estirpes foram identificadas por meio do sequenciamento parcial dos genes marcadores taxonômicos rpoD, gyrB e 16S rRNA. Adicionalmente, foi determinada a produção de ramnolipídeos por cada estirpe através da quantificação do rendimento e, subsequentemente, foi estabelecida a sua composição em congéneres. O capítulo inclui uma avaliação físico-química dos ramnolipídeos produzidos pelas três estirpes de P. aeruginosa, incluindo o seu potencial como emulsificantes e como agentes redutores da tensão superficial. As suas atividades biológicas foram também avaliadas, considerando tanto aplicações ambientais como seu potencial antimicrobiano. Como microrganismos-modelo, foram utilizadas as espécies bacterianas Escherichia coli e Staphylococcus aureus e o fungo

Corynespora cassiicola, um fitopatógeno de importância na cultura da soja.

Palavras-chave: biossurfactantes; caracterização; agente antimicrobiano; interações; biossegurança

SUMMARY

GENERAL INTRODUCTION

The assessment of rhamnolipids, a class of glycolipid biosurfactants predominantly synthesized by *Pseudomonas* spp. (El-Housseiny et al., 2020), has intensified within the scientific community due to their versatile physicochemical characteristics and, more importantly, their wide applicability across multiple fields, including environmental, biomedical, and agricultural applications, among others (Monnier et al., 2018; Adu et al., 2020; Eras-Muñoz et al., 2022; Puyol McKenna et al., 2024). In contrast to conventional synthetic surfactants, which often exhibit low biodegradability and high toxicity, rhamnolipids are considered natural, eco-friendly alternatives with lower toxicity, higher biodegradability compared to synthetic surfactants, and high surface activity, enabling them to effectively reduce and modify surface and interfacial tension (Hogan et al., 2018; Kong et al., 2021; Gidudu and Chirwa, 2022). These attributes have positioned rhamnolipids as attractive molecules aligned with sustainable technologies.

From a biological perspective, rhamnolipids are not merely by-products of microbial metabolism but rather key molecules in microbial ecology, playing central roles in promoting cell motility, regulating biofilm formation, facilitating nutrient assimilation, contributing to virulence, and conferring competitive ecological advantages (Davey, Caiazza, and O'Toole, 2003; Caiazza, Shanks, and O'Toole, 2005). Given their importance, rhamnolipid biosynthesis is tightly regulated and depends on several factors, including quorum sensing and carbon source availability (Abdel-Mawgoud, Lépine, and Déziel, 2010; Aguirre-Ramírez et al., 2012).

In environmental contexts, rhamnolipids exhibit strong emulsifying properties as well as notable efficacy in the remediation of hydrophobic pollutant compounds (Nguyen and Sabatini, 2011; Zeng et al., 2018). For instance, over the last decades, researchers have demonstrated the capacity of rhamnolipids to enhance the biodegradation of hydrocarbons in contaminated environments (Zeng et al., 2018). In addition, rhamnolipids have been shown to improve oil recovery in oilfields, and their ability to remove heavy metals from soils and other natural matrices has been extensively investigated (Amani, 2015; Lee and Kim, 2019; Ramesh and Sakthishobana, 2021;

Jiang et al., 2024). These applications are crucial for addressing modern environmental challenges.

In parallel, the biomedical and agricultural relevance of rhamnolipids has expanded considerably. Multiple studies indicate that these biosurfactants possess intrinsic antimicrobial activity against a broad spectrum of pathogenic bacteria and fungi (Sha and Meng, 2016; Alyousif, Al-Tamimi and Al-Luaibi, 2023). Additionally, growing evidence suggests that rhamnolipids may exhibit antitumor and anticancer activities (Rahimi et al., 2019; Adu et al., 2022; Cerqueira dos Santos et al., 2024). Such findings position rhamnolipids as promising biomolecules for the development of alternative therapeutic strategies in oncology.

Considering the importance of rhamnolipids, the present dissertation is composed of two complementary chapters. The first chapter provides a comprehensive review of rhamnolipid biosynthesis, structural diversity, ecological functions, and their environmental and biomedical applications. The second chapter advances this discussion by exploring and characterizing the potential of three *Pseudomonas aeruginosa* strains from the culture collection of the Laboratório de Biotecnologia e Biodiversidade para o Meio Ambiente (*P. aeruginosa* LBBMA 58, LBBMA 79, and LBBMA 92) in environmental, biomedical, and agricultural contexts. Through this integrative approach, the thesis aims to contribute to a deeper understanding of rhamnolipids by elucidating the molecular and ecological principles governing their production and functionality, while also critically examining their biotechnological potential.

REFERENCES

Abdel-Mawgoud, A.M., Lépine, F. and Déziel, E. (2010) “Rhamnolipids: Diversity of structures, microbial origins and roles,” *Applied Microbiology and Biotechnology*, 86(5), pp. 1323–1336. Available at: https://doi.org/10.1007/S00253-010-2498-2,.

Adu, S.A. *et al.* (2020) “Microbial Biosurfactants in Cosmetic and Personal Skincare Pharmaceutical Formulations,” *Pharmaceutics*, 12(11), p. 1099. Available at: https://doi.org/10.3390/PHARMACEUTICS12111099.

Adu, S.A. *et al.* (2022) “Biosurfactants as Anticancer Agents: Glycolipids Affect Skin Cells in a Differential Manner Dependent on Chemical Structure,” *Pharmaceutics*, 14(2), p. 360. Available at: https://doi.org/10.3390/PHARMACEUTICS14020360/S1.

Aguirre-Ramírez, M. *et al.* (2012) “The *Pseudomonas aeruginosa* rmlBDAC operon, encoding dTDP-L-rhamnose biosynthetic enzymes, is regulated by the quorum-sensing transcriptional regulator RhlR and the alternative sigma factor σS,” *Microbiology (Reading, England)*, 158(Pt 4), pp. 908–916. Available at: https://doi.org/10.1099/MIC.0.054726-0.

Alyousif, N.A., Al-Tamimi, W.H. and Al-Luaibi, Y.Y.Y. (2023) “Antimicrobial and Antioxidant Activity of Rhamnolipids Biosurfactant is Produced by *Pseudomonas aeruginosa*,” *Bionatura*, 8(4). Available at: https://doi.org/10.21931/RB/2023.08.04.25.

Amani, H. (2015) “Study of enhanced oil recovery by rhamnolipids in a homogeneous 2D micromodel,” *Journal of Petroleum Science and Engineering*, 128, pp. 212–219. Available at: https://doi.org/10.1016/J.PETROL.2015.02.030.

Caiazza, N.C., Shanks, R.M.Q. and O’Toole, G.A. (2005) “Rhamnolipids modulate swarming motility patterns of *Pseudomonas aeruginosa*,” *Journal of bacteriology*, 187(21), pp. 7351–7361. Available at: https://doi.org/10.1128/JB.187.21.7351-7361.2005.

Cerqueira dos Santos, S. *et al.* (2024) “Production and characterization of rhamnolipids by *Pseudomonas aeruginosa* isolated in the Amazon region, and potential antiviral, antitumor, and antimicrobial activity,” *Scientific Reports*, 14(1), pp. 1–13. Available at: https://doi.org/10.1038/S41598-024-54828-W;SUBJMETA.

Davey, M.E., Caiazza, N.C. and O’Toole, G.A. (2003) “Rhamnolipid Surfactant Production Affects Biofilm Architecture in *Pseudomonas aeruginosa* PAO1,” *Journal of Bacteriology*, 185(3), p. 1027. Available at: https://doi.org/10.1128/JB.185.3.1027-1036.2003.

El-Housseiny, G.S. *et al.* (2020) “Structural and Physicochemical Characterization of Rhamnolipids produced by *Pseudomonas aeruginosa* P6,” *AMB Express*, 10(1), pp. 1–12. Available at: https://doi.org/10.1186/S13568-020-01141-0/FIGURES/5.

Eras-Muñoz, E. *et al.* (2022) “Microbial biosurfactants: a review of recent environmental applications,” *Bioengineered*, 13(5), p. 12365. Available at: https://doi.org/10.1080/21655979.2022.2074621.

Gidudu, B. and Chirwa, E.M.N. (2022) “Evaluation of the toxicity of a rhamnolipid biosurfactant for its application in the optimization of the bio-electrokinetic remediation of petrochemical contaminated soil,” *Cleaner Engineering and Technology*, 9, p. 100521. Available at: https://doi.org/10.1016/J.CLET.2022.100521.

Hogan, D.E. *et al.* (2018) “Biodegradability and toxicity of monorhamnolipid biosurfactant diastereomers,” *Journal of hazardous materials*, 364, p. 600. Available at: https://doi.org/10.1016/J.JHAZMAT.2018.10.050.

Jiang, M. *et al.* (2024) “Isolation and Characterization of Biosurfactant-Producing Bacteria for Enhancing Oil Recovery,” *Processes*, 12(11), p. 2575. Available at: https://doi.org/10.3390/pr12112575.

Kong, S. *et al.* (2021) “Rhamnolipids Sustain Unchanged Surface Activities during Decomposition in Alkaline Solutions,” *ACS Omega*, 6(24), pp. 15750–15755. Available at: https://doi.org/10.1021/ACSOMEGA.1C01099.

Lee, A. and Kim, K. (2019) “Removal of Heavy Metals Using Rhamnolipid Biosurfactant on Manganese Nodules,” *Water, Air, and Soil Pollution*, 230(11), pp. 1–9. Available at: https://doi.org/10.1007/S11270-019-4319-2/FIGURES/10.

Monnier, N. *et al.* (2018) “Rhamnolipids from *Pseudomonas aeruginosa* are elicitors triggering Brassica napus protection against Botrytis cinerea without physiological disorders,” *Frontiers in Plant Science*, 9, p. 387850. Available at: https://doi.org/10.3389/FPLS.2018.01170/BIBTEX.

Nguyen, T.T. and Sabatini, D.A. (2011) “Characterization and Emulsification Properties of Rhamnolipid and Sophorolipid Biosurfactants and Their Applications,” *International Journal of Molecular Sciences*, 12(2), p. 1232. Available at: https://doi.org/10.3390/IJMS12021232.

Puyol McKenna, P. *et al.* (2024) “Microbial Biosurfactants: Antimicrobial Activity and Potential Biomedical and Therapeutic Exploits,” *Pharmaceuticals*, 17(1), p. 138. Available at: https://doi.org/10.3390/PH17010138.

Rahimi, K. *et al.* (2019) “Cytotoxic effects of mono- and di-rhamnolipids from *Pseudomonas aeruginosa* MR01 on MCF-7 human breast cancer cells,” *Colloids and Surfaces B: Biointerfaces*, 181, pp. 943–952. Available at: https://doi.org/10.1016/J.COLSURFB.2019.06.058.

Ramesh, M. and Sakthishobana, K. (2021) “Significance of biosurfactants in oil recovery and bioremediation of crude oil,” *Green Sustainable Process for Chemical*

and Environmental Engineering and Science: Biosurfactants for the Bioremediation of Polluted Environments, pp. 211–226. Available at: https://doi.org/10.1016/B978-0-12-822696-4.00006-1.
Sha, R. and Meng, Q. (2016) “Antifungal activity of rhamnolipids against dimorphic fungi,” *The Journal of general and applied microbiology*, 62(5), pp. 233–239. Available at: https://doi.org/10.2323/JGAM.2016.04.004.

Zeng, Z. *et al.* (2018) “Mechanisms for rhamnolipids-mediated biodegradation of hydrophobic organic compounds,” *Science of The Total Environment*, 634, pp. 1–11. Available at: https://doi.org/10.1016/J.SCITOTENV.2018.03.349.

CHAPTER I - FROM MICROBIAL SURVIVAL TO HUMAN BENEFIT: A COMPREHENSIVE REVIEW OF RHAMNOLIPID PRODUCTION AND APPLICATIONS.

Abstract

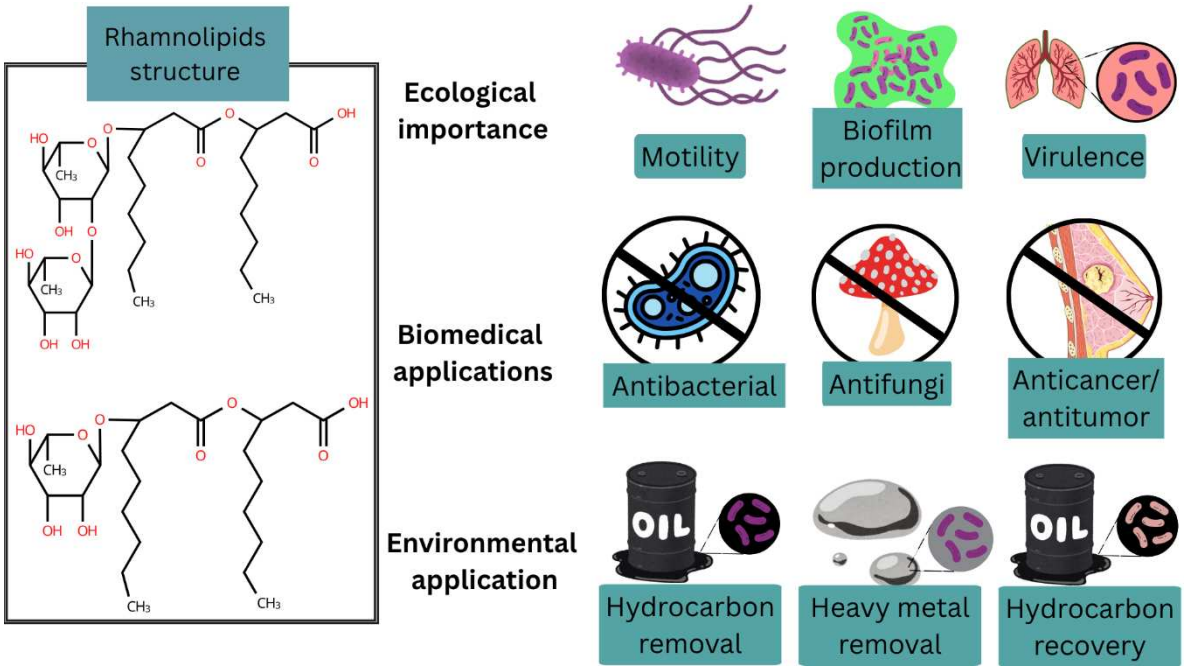
Rhamnolipids are glycolipid biosurfactants of growing interest due to their surface tension–lowering capacity, which is comparable to that of synthetic surfactants, combined with superior biodegradability, low toxicity, minimal environmental impact, and broad functional versatility. These molecules are biosynthesized by species belonging to different bacterial genera, most notably the genus *Pseudomonas*. The presence of rhamnolipids facilitates several key microbial activities, and they are considered essential molecules in microbial ecology and physiology. These activities include promoting motility, supporting colony formation, regulating biofilm development, and enhancing virulence, all of which contribute to microbial survival and improved fitness.

Additionally, rhamnolipids have been shown to enhance hydrocarbon biodegradation, facilitate heavy metal removal, and improve oil recovery efficiency. In the biomedical and agricultural fields, these molecules exhibit a range of beneficial actions, including effective broad-spectrum antimicrobial activity and antibiofilm properties. Furthermore, rhamnolipids show promising therapeutic potential due to reported anticancer and antitumor effects.

However, their cytotoxicity toward non-target cells, hemolytic potential, and variability in biological performance remain major concerns. In addition, large-scale production is constrained by high production costs and the pathogenic nature of some *Pseudomonas* producers, reinforcing the need for alternative non-pathogenic strains and optimized bioprocesses. Overall, these naturally produced compounds offer significant promise for applications in both environmental remediation and therapeutic development. Nevertheless, further efforts are required to develop safer and more efficient production strategies and to fully elucidate the mechanisms underlying the

antimicrobial, anticancer, and antitumor activities of rhamnolipids before their widespread implementation.

Graphical abstract



Source: authors.

Keywords: biosurfactants; biodegradability; bioremediation; antimicrobial activity; anticancer potential.

Introduction

Surface-active agents, commonly referred to as surfactants, are amphiphilic compounds characterized by a unique structural composition that combines both a hydrophilic head and a hydrophobic tail. This structure allows interaction with non-polar substances through the hydrophobic moiety and with aqueous molecules through the hydrophilic head. Due to this amphiphilic nature, surfactants tend to accumulate at liquid interfaces, where they reduce surface tension and modify interfacial behavior (Alwadani and Fatehi, 2018; Yang et al., 2020). A surfactant is typically considered effective when it reduces the surface tension of water from 72 mN m^{-1} to approximately 35 mN m^{-1} (Mulligan, 2005). While many biosurfactants are capable of lowering

surface tension, only a few are regarded as particularly efficient. For instance, surfactins and rhamnolipids can reduce surface tension to values as low as approximately 26 mN m^{-1} (Mulligan, Mudhoo and Sharma, 2014; Liu et al., 2015; Safari et al., 2023). Their amphiphilic structure also facilitates the emulsification and solubilization of hydrophobic organic compounds, as well as micelle formation through self-assembly (Yang et al., 2020).

Depending on their origin, surfactants can be classified as (i) synthetic surfactants or (ii) biosurfactants (Hsu et al., 2025). At present, due to their cost-effectiveness and large-scale petrochemical manufacturing, synthetic surfactants dominate the global market, with an annual production exceeding 15 million tons. Synthetic surfactants are commonly subdivided into four major groups: non-ionic, anionic, cationic, and amphoteric (Hsu et al., 2025). Despite their widespread use, most synthetic surfactants raise environmental and health concerns. Several of these compounds are toxic to living organisms and can disrupt essential biological processes (Johnson et al., 2021). In aquatic systems, surfactants may form surface films that interfere with evaporation processes and alter the water cycle (Luketich, Samouei and Nasrabadi, 2024). Additionally, the release of synthetic surfactants into aquatic ecosystems has been reported to significantly reduce dissolved oxygen levels (Effendi et al., 2017).

The environmental impact of synthetic surfactants is further intensified by two major factors: many of these compounds exhibit slow degradation rates, and during their breakdown, they often generate toxic intermediates that further contaminate the surrounding environment (Johnson et al., 2021). In response to these risks, regulatory agencies have classified certain synthetic surfactants, particularly anionic surfactants, as organic pollutants and have imposed restrictions on their discharge. For example, in Brazil, the maximum permissible concentration of anionic surfactants in freshwater is limited to 0.5 mg L^{-1} (CONAMA, 2005).

In contrast, biosurfactants are microbial by-products considered environmentally friendly alternatives to synthetic surfactants due to their low toxicity, high biodegradability, and production from renewable raw materials. Among them, rhamnolipids exhibit physicochemical properties comparable or superior to those of synthetic surfactants and display inherent structural diversity that broadens their potential applications (Sarubbo et al., 2022). The physicochemical properties of

biosurfactants depend on several factors, one of the most fundamental being molecular weight. Accordingly, biosurfactants are generally classified as either high-molecular-weight (HMW) or low-molecular-weight (LMW) compounds, which exhibit distinct functional characteristics (Sarubbo et al., 2022). HMW biosurfactants typically have molecular masses exceeding 45,000 g mol^{-1} and show high potential as bioemulsifiers. This group includes lipopolysaccharides (e.g., emulsan and liposan), protein–polysaccharide complexes (e.g., alasan), and other polymeric surfactants (Mulligan, Mudhoo and Sharma, 2014; Johnson et al., 2021). In contrast, LMW biosurfactants generally range from 650 to 1,126.4 g mol^{-1} and are more effective at reducing surface tension (Johnson et al., 2021).

The low-molecular-weight (LMW) biosurfactants are chemically classified into three main subclasses: glycolipids, lipopeptides, and phospholipids. Glycolipids have been extensively studied in the last years, improving the understanding about the structure and function of this group. Among the glycolipids, rhamnolipids are of particular interest, due to their potential to be used in several environmental, agricultural and medical fields (Mulligan, Mudhoo and Sharma, 2014).

Structure, Physicochemical Properties, and Biosynthesis of Rhamnolipids

Rhamnolipids are glycolipids structurally defined by the presence of one or two rhamnose molecules linked to a hydrophobic fatty acid tail. This hydrophobic moiety consists of one or two β-hydroxy fatty acid chains (typically containing 8 to 16 carbon atoms) that are interconnected via an ester bond between the β-hydroxyl group of one chain and the carboxyl group of the other (Abdel-Mawgoud, Lépine and Déziel, 2010; Liu et al., 2018). Variations in their degree of unsaturation, chain length, and whether the rhamnose moiety is present as a mono- or di-rhamnose structure directly influence their hydrophobicity and physicochemical characteristics. Currently, over sixty distinct rhamnolipid congeners have been identified. Among them, the most common and abundant species are Rha–Rha–C10–C10 and Rha–C10–C10 (Hošková et al., 2015).

Although a broad range of microorganisms is capable of synthesizing rhamnolipids, *Pseudomonas aeruginosa* occupies a unique position. It was the first bacterium reported to produce rhamnolipids and remains the most extensively studied producer to date (Abdel-Mawgoud, Lépine and Déziel, 2010). Other microorganisms reported in

the literature as potential rhamnolipid producers include species of the genus *Burkholderia*, such as *B. thailandensis*, *B. plantarii*, and *B. pseudomallei* (Häußler et al., 2003; Hörmann et al., 2010; Kumar et al., 2023), as well as representatives of *Pseudoxanthomonas*, *Acinetobacter calcoaceticus*, and certain strains of *Klebsiella pneumoniae* (Purwasena, Astuti and Utami, 2020; Ahmad et al., 2021; Zhu et al., 2022).

More recently, the identification of rhamnolipid production in marine bacteria belonging to genera such as *Marinobacter* and *Halomonas* has revealed a broader phylogenetic diversity among producing organisms than previously recognized (Donio et al., 2013; Tripathi et al., 2019). However, although the taxonomic diversity and biotechnological potential of these strains are increasingly acknowledged, the genetics underlying rhamnolipid biosynthesis remain poorly characterized in many of them. A deeper understanding of this genetic control is crucial, as it would elucidate regulatory and biosynthetic mechanisms and enable the development of strategies to optimize rhamnolipid production. At present, *Pseudomonas aeruginosa* remains the best-studied model organism and has therefore been extensively investigated at the genetic level, revealing a sophisticated and complex regulatory network linking metabolism, quorum sensing, and environmental responses.

Rhamnolipid biosynthesis in *Pseudomonas aeruginosa* is a well-characterized genetic pathway that integrates both metabolic enzymes and a complex regulatory network. It is known that rhamnolipid production requires two distinct types of precursors: one for the hydrophilic sugar moiety and another for the hydrophobic lipid moiety (Dobler et al., 2016).

Pseudomonas aeruginosa can utilize a wide variety of carbon sources. Although its preferred substrates are amino acids and organic acids (Rojo, 2010), it is a metabolically versatile organism that can also grow on alternative carbon sources such as glucose, olive oil, and glycerol (Dobler et al., 2016). Some of these compounds are metabolized to generate intermediates that can be readily redirected toward the formation of glucose-6-phosphate. Through the sequential activity of enzymes encoded by the *rmlBDAC* operon, glucose-6-phosphate is progressively converted into deoxythymidine diphosphate (dTDP)-L-rhamnose. This pathway involves multiple enzymatic steps, including the action of AlgC and RmlA (Figure 1), among others,

ensuring the supply of activated rhamnose units required for rhamnolipid assembly (Dobler et al., 2016; Tan and Li, 2018).

As mentioned previously, the biosynthesis of rhamnolipids requires the generation of a hydrophobic fraction. For this purpose, it is necessary to produce a molecule derived from central metabolic pathways: acyl-coenzyme A (acyl-CoA). Acyl-CoA can be formed either from glucose through gluconeogenic pathways or via the catabolism of fatty acids through β-oxidation (Dobler et al., 2016). In the case of β-oxidation, once acyl-CoA is obtained, it becomes the main substrate for the type II fatty acid synthase pathway (FAS II) (Chong and Li, 2017a, 2017b). During this process, acyl-CoA is elongated and reduced to yield a β-hydroxyacyl–acyl carrier protein (ACP) intermediate.

At this stage, the product of the *rhlA* gene becomes essential for the continuation of rhamnolipid biosynthesis. This gene encodes the enzyme 3-(3-hydroxyalkanoyloxy)alkanoate synthase, which redirects β-hydroxyacyl-ACP intermediates away from the conventional fatty acid synthesis pathway, promoting the formation of 3-(hydroxyalkanoyloxy)alkanoic acids (HAAs). These compounds subsequently serve as the direct hydrophobic precursors of rhamnolipids (Tan and Li, 2018; Schellenberger et al., 2021).

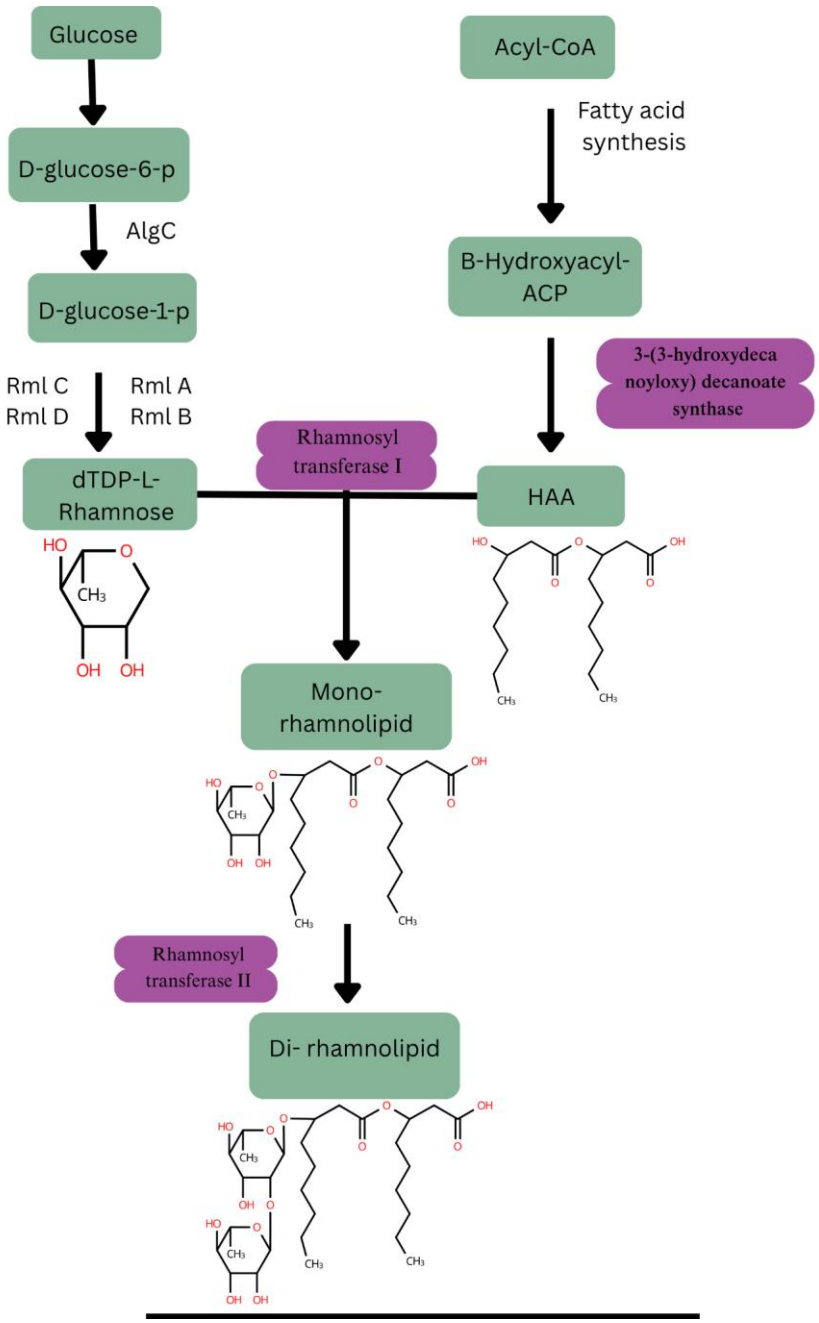
Interestingly, HAAs not only play a structural role in rhamnolipid biosynthesis but also exhibit several biological properties, including the promotion of swarming motility, surface wetting, and biofilm development (Davey, Caiazza and O'Toole, 2003a; Caiazza, Shanks and O'Toole, 2005). Thus, HAAs have a dual function in *Pseudomonas aeruginosa*, acting both as signaling molecules within microbial communities and as building blocks for the subsequent assembly of rhamnolipids (Davey, Caiazza and O'Toole, 2003a).

Following the synthesis of both precursors—the hydrophobic HAA and the hydrophilic dTDP-L-rhamnose—the assembly of the glycolipid begins. The first glycosylation step is catalyzed by rhamnosyltransferase I, encoded by the *rhlB* gene. This enzyme facilitates the transfer of a rhamnose moiety to the HAA, resulting in the formation of mono-rhamnolipids (Dobler et al., 2016). However, the process does not necessarily end at this stage. Rhamnosyltransferase II, encoded by the *rhlC* gene, catalyzes the

addition of a second rhamnose moiety, leading to the production of di-rhamnolipids (Chong and Li, 2017b).

This two-step enzymatic process results in the formation of amphiphilic rhamnolipid molecules, composed of a lipid tail linked to a sugar head group (Figure 1). It is important to note that the proportion of mono- and di-rhamnolipids produced is not fixed; rather, the relative abundance of these congeners is determined by a complex interplay of genetic and environmental factors.

Figure 1. Metabolic pathway involved in rhamnolipid biosynthesis

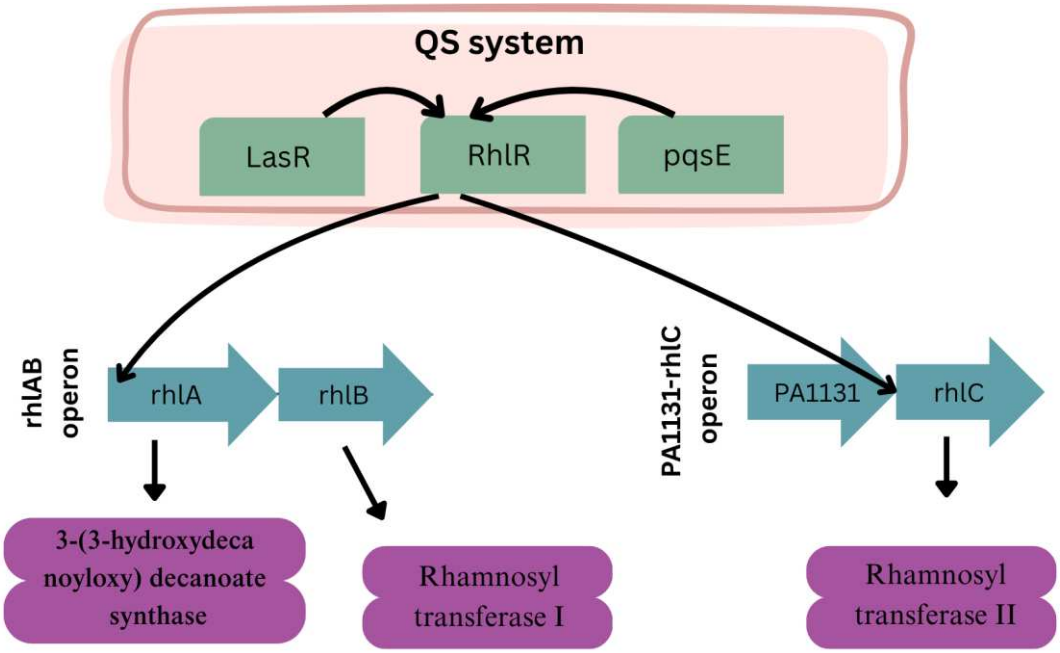


From an enzymatic perspective, the formation of mono- and di-rhamnolipids is driven by the activity of two rhamnosyltransferases, RhlB and RhlC. In the case of RhlB, as described previously, this enzyme catalyzes the transfer of a rhamnose unit to HAAs, resulting in the formation of mono-rhamnolipids. Subsequently, RhlC introduces a second rhamnose moiety, leading to the production of di-rhamnolipids (Rahim et al., 2001; Dobler et al., 2016; Chong and Li, 2017b). Consequently, conditions that favor *rhlC* expression promote higher proportions of di-rhamnolipids. In contrast, conditions associated with reduced *rhlC* expression lead to decreased di-rhamnolipid synthesis and, as a result, to the accumulation of mono-rhamnolipids (Rahim et al., 2001).

The growth phase of the culture has a significant impact on the structural composition of rhamnolipids. It has been observed that during the early exponential phase, mono-rhamnolipids predominate, reflecting the initial activity of RhlB. As the culture progresses into the stationary phase, the relative abundance shifts toward di-rhamnolipid synthesis, coinciding with increased expression of *rhlC*. This transition is tightly regulated by quorum sensing (QS). The expression of both *rhlB* and *rhlC* depends on regulation by the *las* and *rhl* QS systems (Figure 2), which respond to increasing cell density through the accumulation of N-acyl-homoserine lactones (AHLs).

Activation of the quorum sensing network not only coordinates rhamnolipid biosynthesis with the production of other virulence-associated molecules (Aguirre-Ramírez et al., 2012), but also ensures that the energetically costly synthesis of surface-active compounds occurs preferentially at high population densities. Under these conditions, cooperative behaviors such as biofilm development and defense against potential competitors confer a selective advantage to individual members of the *Pseudomonas aeruginosa* population.

Figure 2. Quorum sensing network regulating the expression of rhamnolipid biosynthesis genes.



However, quorum sensing (QS) is not the only variable that can influence rhamnolipid synthesis. The relative proportions of mono- and di-rhamnolipids are also directly regulated by the composition of the culture medium (Abdel-Mawgoud, Lépine and Déziel, 2010). Carbon sources containing long-chain fatty acids tend to favor mono-rhamnolipid production, whereas more hydrophilic substrates, such as glucose or glycerol, may enhance di-rhamnolipid synthesis (Nicolò et al., 2017). In addition, the final rhamnolipid composition is strain-dependent, varying according to the specific bacterial strain used (Abdel-Mawgoud, Lépine and Déziel, 2010). This inherent variability is a critical consideration for the targeted synthesis of these biosurfactants.

Taken together, it can be concluded that the predominance of mono- or di-rhamnolipids is not fixed but is strongly influenced by gene regulation, substrate availability, culture conditions, and strain-specific factors.

Functional roles of rhamnolipids in microbial physiology and ecology

Rhamnolipids possess unique physicochemical characteristics due to their amphiphilic nature, which confer the capacity to reduce surface tension and stabilize emulsions.

Beyond these properties, they also play multiple roles in the physiology and ecology of producing bacteria, providing advantages that are essential for survival, colonization, and environmental interactions (Davey, Caiazza and O'Toole, 2003b; Caiazza, Shanks and O'Toole, 2005; Abdel-Mawgoud, Lépine and Déziel, 2010).

One of the best-characterized functions of rhamnolipids is their involvement in bacterial motility. Their chemical structure reduces surface tension at solid–liquid interfaces, allowing flagellated cells to migrate (Caiazza, Shanks and O'Toole, 2005; Glick et al., 2010). Rhamnolipids also promote sliding motility, a flagella-independent surface movement considered a passive process that relies on the wetting activity of biosurfactants (Abdel-Mawgoud, Lépine and Déziel, 2010). Thus, rhamnolipids directly contribute to surface colonization by facilitating the movement and expansion of bacterial populations.

In recent years, it has become clear that the same factors influencing swarming motility also affect other bacterial behaviors, including biofilm formation. As previously described, rhamnolipids promote and facilitate bacterial migration, enabling the initial formation of microcolonies and contributing to the structural differentiation of biofilms (Pamp and Tolker-Nielsen, 2007). Beyond their role in the early stages of biofilm development, rhamnolipids are also involved in the maintenance and remodeling of biofilm architecture. Early work by Davies et al. (1998) demonstrated that *Pseudomonas aeruginosa* mutants deficient in *lasI*, a gene involved in quorum sensing (QS), were unable to form differentiated biofilms, instead producing structures lacking characteristic water channels. As a result, these biofilms were more susceptible to biocides such as sodium dodecyl sulfate (SDS). Subsequently, Davey, Caiazza and O'Toole (2003b) provided direct evidence that rhamnolipids maintain open channels by modulating cell–cell interactions and bacterial attachment to surfaces during later stages of biofilm development, when quorum sensing is fully induced.

The importance of these channels lies in their role as conduits for nutrient distribution, waste removal, and signal diffusion throughout the biofilm, thereby ensuring metabolic activity and communication even at later stages of development (Quan et al., 2022). In their absence, biofilms collapse, forming uniform mats that lack internal nutrient circulation and consequently exhibit reduced community viability. Additional support for this concept comes from studies involving *rhlA* mutants. This gene encodes the

enzyme 3-(3-hydroxyalkanoyloxy)alkanoate synthase, which is essential for the production of the hydrophobic moiety of rhamnolipids. Accordingly, although these mutants were able to initiate channel formation around microcolonies, they failed to maintain these structures beyond six days, ultimately forming homogeneous cell layers (Davey, Caiazza and O'Toole, 2003b).

Conversely, biofilm development can also be disrupted by an excess of rhamnolipids in the surrounding environment (Davey, Caiazza and O'Toole, 2003b), demonstrating that tight regulation of rhamnolipid levels is crucial for biofilm maturation and final architecture.

Pseudomonas aeruginosa is considered an opportunistic pathogen and is a common cause of healthcare-associated infections, particularly pneumonia (Reynolds and Kollef, 2021). This bacterium can cause severe lung damage when it establishes chronic infections in its host. However, the infection process depends on multiple extracellular products, and rhamnolipids have been identified as key virulence determinants. Clinical studies have demonstrated a correlation between the severity of *P. aeruginosa* lung infections and rhamnolipid concentrations in patients (Kownatzki, Tümmler and Döring, 1987).

Early studies showed that rhamnolipids are heat-stable hemolytic molecules, particularly those containing two rhamnose moieties (Fujita, Akino and Yoshioka, 1988; McClure and Schiller, 1992). Notably, *P. aeruginosa* is not the only species capable of producing such compounds. Certain strains of *Burkholderia pseudomallei* and *B. plantarii* have also been reported to synthesize rhamnolipid congeners with chemical structures similar to those produced by *P. aeruginosa*, which are capable of lysing erythrocytes and exhibiting cytotoxic activity (Häußbler et al., 2003). In the case of *B. plantarii*, rhamnolipids have additionally been shown to exert immunostimulatory effects by promoting the production of tumor necrosis factor alpha and, when present at high concentrations, may contribute to sepsis and septic shock syndrome (Andrä et al., 2006).

Beyond their role in host-pathogen interactions, rhamnolipids are also central to the competitive strategies employed by P. aeruginosa against other microorganisms in polymicrobial environments. They function as direct antimicrobial agents by disrupting

cell membranes and penetrating the peptidoglycan layer, ultimately causing cell death, while also contributing to the dispersal of biofilms formed by competing species. In addition, rhamnolipids can form micelles that facilitate the transport of toxic metabolites. Through these combined mechanisms—membrane interaction, penetration of the peptidoglycan, and biofilm disruption—rhamnolipids exert multifaceted inhibitory effects that support resource competition and defense against competing microorganisms (Gdaniec et al., 2022).

Although these activities are often regarded as virulence-associated traits and, consequently, described as undesirable characteristics, they also reflect important ecological advantages that contribute to the success of *P. aeruginosa* in a wide range of environments that support its growth. Thus, rhamnolipids should not be viewed merely as metabolic by-products but rather as multifunctional molecules that bridge pathogenicity and ecological fitness.

Environmental applications

The amphiphilic nature of rhamnolipids, common to all surfactants, arises from their molecular structure, which combines hydrophilic sugar head groups with hydrophobic fatty acid tails. The specific properties of each congener, such as its affinity for water or oil, are determined by the length and degree of saturation of the hydrophobic tail and by the number of rhamnose units present. This structural versatility allows rhamnolipids to be tailored for specific applications across diverse sectors, including pharmaceuticals, cosmetics, environmental remediation, and agriculture.

In recent years, the popularity of rhamnolipids has increased within the environmental and biotechnological fields due to their capacity to act as eco-friendly alternatives to synthetic surfactants. Their ability to form stable micelles, reduce surface tension, and emulsify hydrophobic compounds has led to their successful application in various remediation processes. In particular, their roles in hydrocarbon biodegradation (Liu et al., 2018), heavy metal removal (Dahrazma and Mulligan, 2007; Khiyavi et al., 2020), enhanced oil recovery (Al-Wahaibi et al., 2014; Wu et al., 2022b), and wastewater treatment (Sonmez, Akarsu and Sivri, 2025) have been well documented.

1.4.1 Bioremediation of Hydrocarbons and other Organic Pollutants

The use of rhamnolipids in bioremediation relies on their ability to reduce surface and interfacial tension, which in turn enhances the bioavailability of hydrophobic organic compounds to degrading microorganisms (Berkat et al., 2025; Rather et al., 2025). In soils and sediments contaminated with petroleum derivatives, these biosurfactants alter bacterial attachment to geomedia and their transport behavior within soil pores by modifying electrostatic, hydrophobic, and steric interactions between bacterial cells and sediment particles (Zhong et al., 2016). Increased bioavailability allows microbial communities to access previously inaccessible substrates (Kaczorek et al., 2018), thereby accelerating biodegradation rates and shortening remediation times (Chen et al., 2020). In this context, microorganisms often synthesize rhamnolipids to solubilize hydrophobic substrates, facilitating their use as carbon sources (Eras-Muñoz et al., 2022) and ultimately improving microbial fitness. Studies conducted over the last five years have confirmed that, even at low concentrations, rhamnolipids can significantly enhance the degradation efficiency of long-chain hydrocarbons, polycyclic aromatic hydrocarbons, and lubricating oils (Wu et al., 2022a; Gaur et al., 2023; Luo et al., 2025).

Beyond ex situ remediation, rhamnolipids also show potential for in situ applications, although with a less extensive track record. A representative example is their use in microbial enhanced oil recovery (MEOR), where rhamnolipid injection into reservoirs enhances crude oil recovery by altering rock wettability and reducing oil–water interfacial tension (Purwasena, Astuti and Utami, 2020; Wu et al., 2022b). The main advantages of MEOR include a low environmental footprint, as it avoids secondary pollution, and its suitability for repeated field applications (Wu et al., 2022b). Despite promising results in specific cases (Al-Wahaibi et al., 2014), a comprehensive understanding of the efficacy and potential limitations of in situ rhamnolipid applications requires further investigation. Moreover, the translation of laboratory-scale results to full-scale field applications remains constrained by the high costs associated with large-volume rhamnolipid production and by the physicochemical variability of natural environments (Henkel et al., 2012).

In addition, the structural diversity of rhamnolipid congeners—comprising mono- and di-rhamnolipids with varying fatty acid chain lengths—suggests that their functional performance is not universal (Costa et al., 2018). Consequently, optimization

strategies are likely to enhance rhamnolipid yield and effectiveness only for specific strains and defined environmental conditions, and may be less effective under alternative scenarios.

Although rhamnolipids are generally regarded as environmentally friendly molecules, their ecological impact on plants warrants careful consideration. A study by Millioli et al. (2009) demonstrated a clear dose-dependent phytotoxic response in the model species *Lactuca sativa*. At low concentrations (1 mg g^{-1}), rhamnolipids slightly increased the germination index; however, as concentrations increased, germination was progressively inhibited. At concentrations of 10 and 15 mg g^{-1}, germination was reduced by approximately 80% relative to the control. This phytotoxic effect is likely attributable to the same membrane-disrupting mechanism that underpins their antimicrobial activity. Rhamnolipids can integrate into and solubilize the lipid bilayers of plant root cells, leading to increased membrane permeability, loss of essential nutrients and ions, and ultimately, cell death. These findings indicate that the environmental impact of biosurfactants is not fully understood and can be strongly concentration dependent. Therefore, environmental risk assessments should be integrated into bioremediation studies and other field-scale applications to minimize potential adverse effects.

1.4.2 Heavy-Metal Removal and Soil Washing Processes

For centuries, heavy metals were considered indispensable materials due to their wide applicability across multiple sectors, including energy, agriculture, medicine, and even cosmetics (Arshad et al., 2020; Zhou et al., 2023). However, over recent decades, heavy metals have been extensively investigated, greatly expanding our understanding of their environmental and biological impacts. Currently, their high toxicity and bioaccumulation potential make heavy metal contamination a critical global problem affecting all forms of life, including humans, animals, plants, and microorganisms. Once accumulated in living organisms, heavy metals can interfere with vital biochemical processes due to their disruptive nature, leading to various diseases or, in severe cases, death (Arshad et al., 2020; Angon et al., 2024).

Although numerous physicochemical and biological approaches have been proposed in recent years to reduce heavy metal concentrations in water and soil, their effectiveness is often limited. In addition to low efficiency, many of these methods are costly and impractical for field-scale applications. Moreover, conventional removal

techniques frequently generate secondary pollution, the environmental impact of which may be comparable to or even exceed that caused by the heavy metals themselves. Considering these limitations, the development of environmentally safe, efficient, and cost-effective methodologies for heavy metal remediation has become critically important.

Beyond their role in hydrocarbon biodegradation, rhamnolipids have also been investigated for their notable metal-chelating properties (Sorour et al., 2025). These biosurfactants are capable of sequestering toxic metals from contaminated soils and sediments, making rhamnolipids promising candidates for application in complex environmental remediation processes.

In recent years, soil-washing techniques have emerged as strategic approaches for heavy metal removal. These processes typically employ synthetic chelators or mineral acids; however, rhamnolipids have also been explored as alternative washing agents. It has been demonstrated that the addition of biosurfactants can significantly improve the removal efficiency of heavy metals such as cadmium, in some cases achieving removal rates exceeding 90% (Lima et al., 2011). In these systems, rhamnolipid solutions are circulated through contaminated matrices to mobilize and extract adsorbed metals (Dahrazma and Mulligan, 2007; Lima et al., 2011). Compared with mineral acids and synthetic chelators, biosurfactant-based washing solutions offer several advantages: they are less corrosive, more biodegradable, and pose a lower risk of secondary contamination. Nevertheless, metal removal efficiency remains highly dependent on factors such as soil pH, organic matter content, and ionic competition within the matrix (Wuana et al., 2011; Kou et al., 2024).

Heavy metal removal using rhamnolipids has primarily been evaluated under ex situ conditions, with encouraging results. However, scaling these approaches to industrial or field levels remains costly, and information regarding potential side effects in complex environmental systems is still limited. Despite these challenges, the capacity of rhamnolipids to simultaneously mobilize both heavy metals and hydrophobic organic compounds represents a unique and highly valuable characteristic. This dual functionality is particularly advantageous for sites with mixed contamination, where conventional remediation methods often fail. Consequently, rhamnolipids emerge as promising multifunctional agents with significant potential for diverse environmental applications.

Biomedical applications of Rhamnolipids

Rhamnolipids have been studied primarily for their capacity as industrial surfactants. However, in recent years, their biomedical and veterinary potential has gained increasing attention due to their biological effects, particularly their anticancer and antitumor potential, antimicrobial activity, and ability to disrupt biofilms (Rahimi et al., 2019; Buonocore et al., 2023; Malakar et al., 2024). Several bacterial species have naturally evolved to synthesize rhamnolipids with the primary objective of facilitating microbial interactions; however, this is not their only function. These versatile molecules exhibit a broad spectrum of biological activities that interfere with essential cellular mechanisms and ultimately inhibit growth or induce cell death in both bacteria and fungi (Buonocore et al., 2023; Malakar et al., 2024; Touabi et al., 2025).

These effects derive from the amphiphilic nature of rhamnolipids, which allows them to interact with lipid membranes. The anchoring of rhamnolipids to cell membranes increases membrane permeability, leading to disruption of ionic balance and triggering a cascade of events that can ultimately result in cell destruction (Shao et al., 2017; Potapov et al., 2023).

1.5.1 Rhamnolipids as potential Anticancer and Antitumor Agents.

Although rhamnolipids have primarily gained attention for their antimicrobial activity, in recent years they have also attracted interest due to their promising anticancer and antitumor effects. Over the past two decades, several studies have demonstrated that these glycolipids exhibit cytotoxic activity against a range of malignant cell lines (Semkova et al., 2021; Adu et al., 2022; Ankulkar et al., 2022). These findings position rhamnolipids as potential bio-derived compounds with possible applications in therapeutic oncology.

One of the earliest reports describing the anticancer potential of rhamnolipids was provided by Thanomsub et al. (2006), who observed a significant reduction in the proliferation of human breast cancer cells (MCF-7) at a rhamnolipid concentration of 6.25 μg mL^{-1}. This effect was observed using the di-rhamnolipid congener Rha–Rha–C10–C10 isolated from *Pseudomonas aeruginosa* B189 and subsequently purified by chromatography. These observations were later expanded upon by several researchers. For example, Rahimi et al. (2019) demonstrated the antitumor potential

of rhamnolipids against MCF-7 cells, reporting IC_{50} values of 25.87 μg mL^{-1} and 31.00 μg mL^{-1} for mono- and di-rhamnolipids, respectively.

In addition, several investigations have demonstrated the anticancer potential of rhamnolipids in leukemia cell lines. Ankulkar et al. (2022) reported that the di-rhamnolipid Rha–Rha–C10–C10 exhibited strong cytotoxicity toward the leukemia cell line K-562, achieving complete cell inhibition at a concentration of 66.6 μg mL^{-1}. Similarly, Christova et al. (2013) showed that the addition of mono-rhamnolipids (Rha–C10–C10) at a concentration of 50 μM resulted in approximately 50% loss of cell viability in leukemia cells. In this study, mono-rhamnolipids displayed greater potency than di-rhamnolipids (Rha–Rha–C10–C10).

More recent studies have confirmed the anticancer and antitumor potential of rhamnolipids in other cancer cell lines. Muthaliar, Sevakumaran and Bhubalan (2019) reported cytotoxic effects in HepG2 (liver hepatocellular carcinoma) cells, where the addition of mono-rhamnolipids at a concentration of 100 μg mL^{-1} reduced cell viability by 50%. In contrast, higher concentrations of di-rhamnolipids (150 μg mL^{-1}) were required to achieve a comparable effect. Similarly, Semkova et al. (2021) investigated the anticancer activity of both mono- and di-rhamnolipids in human breast cancer cell lines and demonstrated a pronounced sensitivity, particularly in metastatic MDA-MB-231 cells. In addition, mono-rhamnolipids were consistently more effective than di-rhamnolipids.

The cytotoxic mechanisms of rhamnolipids appear to be multifactorial. According to Semkova et al. (2021), rhamnolipid exposure affects the formation of acidic vesicular organelles (AVOs), potentially triggering pro-apoptotic signaling pathways that ultimately lead to apoptosis. The authors further reported that rhamnolipids can disrupt pre-existing AVOs by inducing membrane permeabilization, resulting in cellular destabilization and activation of apoptotic processes. In both scenarios, the outcome is a reduction in cancer cell viability.

Nevertheless, concerns regarding the therapeutic use of rhamnolipids have been raised in recent years. Jiang et al. (2014) reported that rhamnolipids exhibit comparable cytotoxicity toward both cancerous and non-cancerous cells. In contrast to the findings of Semkova et al. (2021), these authors suggested that rhamnolipid-induced cytotoxicity may be primarily associated with surface tension reduction in the culture medium, acting similarly to chemical surfactants and lacking cellular specificity. This limitation is particularly relevant at higher concentrations and highlights the

potential for off-target effects. Consequently, the precise mechanisms by which rhamnolipids interact with both cancerous and healthy cells remain unclear. It is plausible that the reported anticancer effects are not mediated by a single mechanism but rather by a combination of factors. The membrane-disrupting activity that leads to apoptosis, as proposed by Semkova et al. (2021), and the non-specific reduction in surface tension that affects cell viability, as suggested by Jiang et al. (2014), may coexist. The predominance of one mechanism over the other could depend on critical variables such as the rhamnolipid concentration, the specific congener composition of the extract, and the cell type being targeted. Further molecular, pharmacokinetic, and immunological studies are therefore required to clarify these complex interactions and to establish safe therapeutic windows that allow rhamnolipids to be used without compromising patient health or survival.

Beyond the uncertainty surrounding their mechanisms of action, the translation of rhamnolipids into viable anticancer therapies faces a significant pharmacological hurdle: the route of administration. The intrinsic hemolytic activity of these biosurfactants, particularly at higher concentrations, presents a major challenge for systemic delivery. Intravenous administration, a common route for chemotherapeutic agents, could lead to rapid erythrocyte lysis, resulting in anemia and other severe complications. While topical application might be feasible for certain types of cancers (e.g., skin cancer), it would be ineffective for treating internal, metastatic tumors. Therefore, the development of targeted delivery systems, such as encapsulation in nanoparticles or liposomes, may be essential to shield healthy tissues, including red blood cells, from direct contact with rhamnolipids and to ensure their safe and effective use in oncology. This represents a critical and underexplored area of research.

1.5.2 Antimicrobial and anti-biofilm potential

Rhamnolipids have exhibited effective antibacterial activity against a wide spectrum of microorganisms, successfully inhibiting growth and acting as biocidal agents against both Gram-positive bacteria, such as *Staphylococcus aureus* and *Enterococcus faecium*, and Gram-negative organisms, including *Escherichia coli* (Saadati et al., 2022; Sharaf et al., 2022; Cerqueira dos Santos et al., 2024). As occurs with most antimicrobial agents, rhamnolipids act in a dose-dependent manner, whereby higher concentrations of these biosurfactants result in stronger inhibitory effects. Furthermore, structural differences among rhamnolipid congeners play a crucial role in their biocidal

capacity. Mono-rhamnolipids often exhibit stronger antimicrobial effects than di-rhamnolipids, likely due to their higher lipophilicity (Zhao et al., 2022).

Rhamnolipids can also disrupt biofilm structures by interfering with bacterial adhesion and altering cell surface hydrophobicity (Sen et al., 2020). This activity is particularly relevant in clinical settings, where biofilms confer antibiotic resistance and can facilitate the horizontal transfer of resistance genes (Liu, Prentice and Webber, 2024), thereby further increasing the risk of infections in chronic wounds, implanted medical devices, or pulmonary infections associated with cystic fibrosis. By reducing surface adhesion and promoting cell detachment, rhamnolipids contribute to both biofilm prevention and biofilm eradication.

Despite these advantages, the clinical implementation of rhamnolipids faces important challenges. At higher concentrations, the same membrane-disrupting properties responsible for microbial inhibition can also affect mammalian cell membranes, leading to cytotoxic effects (Xu et al., 2024). In addition, hemolysis assays have shown that certain rhamnolipid congeners are capable of lysing erythrocytes even at intermediate concentrations, with hemolytic activity reported at approximately 35 $mg\ mL^{-1}$ (Haba et al., 2014; Alyousif, Al-Tamimi and Al-Luaibi, 2023). Although this effect is concentration dependent, it raises significant concerns regarding clinical applicability.

Another limitation arises from the variability of physiological conditions. Factors such as pH, ionic strength, and the presence of serum proteins can alter micelle formation and, consequently, biological activity (Lu et al., 2018). For example, the antimicrobial efficiency of rhamnolipids tends to decrease under neutral or alkaline conditions but increases in slightly acidic environments (Freitas Ferreira et al., 2018).

Furthermore, large-scale pharmaceutical production of rhamnolipids remains challenging because their biological activity is highly sensitive to subtle variations in fatty acid chain length, degree of saturation, and number of rhamnose units. Consequently, production requires not only high-purity formulations but also strict control over congener composition to avoid unpredictable variations in biological activity. Achieving this level of control is complex and demands a deep understanding of microbial metabolism, advanced analytical techniques, and sophisticated bioprocess engineering to ensure consistency and safety in both industrial and biomedical applications. These requirements substantially increase production costs and limit commercial viability. Nevertheless, the low risk of resistance development,

combined with biodegradability and broad-spectrum antimicrobial efficacy, continues to motivate extensive research worldwide.

1.5.3 Synergism of Rhamnolipids with Conventional Therapeutics Agents

Due to their ability to increase membrane permeability and disrupt bacterial functions (Saadati et al., 2022), rhamnolipids exhibit promising characteristics that can be exploited synergistically when combined with conventional antibiotics and other antimicrobial agents, thereby reducing the required antibiotic concentration or dose. According to Chang et al. (2023), the combination of rhamnolipids with linezolid results in synergistic inhibition against Enterococcus faecium, leading to a reduction in the minimum inhibitory concentration (MIC) required. Consequently, the combined use of rhamnolipids and antibiotics represents a potentially effective strategy for combating multidrug-resistant strains.

However, at present, studies exploring the interactions between rhamnolipids and existing antibiotics remain limited. As a result, significant knowledge gaps persist regarding the mechanisms underlying these synergistic effects. Furthermore, it remains unclear how rhamnolipids interact with bacterial species beyond the well-studied Staphylococcus aureus and Escherichia coli.

The use of rhamnolipids as adjuvants in antifungal therapies has also attracted increasing attention. When combined with conventional fungicides, rhamnolipids can enhance the diffusion of active ingredients, thereby improving treatment effectiveness. Nevertheless, maintaining selectivity remains a major challenge, as formulations that enhance antimicrobial potency may also exert toxic effects on plants and animals, including humans.

Limitations and Future Perspectives

The widespread adoption of rhamnolipids as therapeutic agents can only occur after overcoming several critical obstacles. First, to establish safe concentrations for use in animals, plants, humans, and the environment, it is essential to conduct comprehensive toxicological assessments that clarify potential side effects under both acute and chronic exposure conditions. Second, the selection of a single producing strain will be necessary to ensure reproducibility of biological activity. As previously discussed, rhamnolipid composition and functionality vary considerably among strains.

In addition, it will be necessary to establish production protocols optimized for specific growth substrates that are readily available and economically viable across different regions.

Furthermore, important ethical and biosafety concerns arise from the dual role of rhamnolipids as both bacterial metabolites and key virulence factors in *Pseudomonas aeruginosa*. For this reason, industrial production using non-pathogenic rhamnolipid-producing strains, such as *Burkholderia thailandensis* or *Pseudomonas putida*, represents a realistic and safer alternative (Funston et al., 2017; Pang et al., 2024).

Finally, the remarkable potential of rhamnolipids to act as anticancer and antitumor agents, as well as their ability to synergize with conventional antibiotics, penetrate resilient biofilms, modulate cell surface interactions, and enhance biodegradation processes, requires more in-depth investigation. A deeper understanding of the mechanisms underlying these interactions, particularly in complex environmental and biological systems, is still needed.

Despite these challenges, rhamnolipids have demonstrated significant value in both clinical and environmental applications. Therefore, a multidisciplinary approach integrating microbiology, chemistry, medicine, and environmental biotechnology is essential to unlock their full potential and ensure their safe and effective use.

REFERENCES

Abdel-Mawgoud, A.M., Lépine, F. and Déziel, E. (2010) “Rhamnolipids: Diversity of structures, microbial origins and roles,” *Applied Microbiology and Biotechnology*, 86(5), pp. 1323–1336. Available at: https://doi.org/10.1007/S00253-010-2498-2,.

Adu, S.A. *et al.* (2022) “Biosurfactants as Anticancer Agents: Glycolipids Affect Skin Cells in a Differential Manner Dependent on Chemical Structure,” *Pharmaceutics*, 14(2), p. 360. Available at: https://doi.org/10.3390/PHARMACEUTICS14020360/S1.

Aguirre-Ramírez, M. *et al.* (2012) “The *Pseudomonas aeruginosa* rmlBDAC operon, encoding dTDP-L-rhamnose biosynthetic enzymes, is regulated by the quorum-sensing transcriptional regulator RhlR and the alternative sigma factor σS,” *Microbiology (Reading, England)*, 158(Pt 4), pp. 908–916. Available at: https://doi.org/10.1099/MIC.0.054726-0.

Ahmad, Z. *et al.* (2021) “Production, functional stability, and effect of rhamnolipid biosurfactant from Klebsiella sp. on phenanthrene degradation in various medium systems,” *Ecotoxicology and Environmental Safety*, 207, p. 111514. Available at: https://doi.org/10.1016/J.ECOENV.2020.111514.

Alwadani, N. and Fatehi, P. (2018) “Synthetic and lignin-based surfactants: Challenges and opportunities,” *Carbon Resources Conversion*, 1(2), pp. 126–138. Available at: https://doi.org/10.1016/J.CRCON.2018.07.006.

Al-Wahaibi, Y. *et al.* (2014) “Biosurfactant production by Bacillus subtilis B30 and its application in enhancing oil recovery,” *Colloids and Surfaces B: Biointerfaces*, 114, pp. 324–333. Available at: https://doi.org/10.1016/J.COLSURFB.2013.09.022.

Alyousif, N.A., Al-Tamimi, W.H. and Al-Luaibi, Y.Y.Y. (2023) “Antimicrobial and Antioxidant Activity of Rhamnolipids Biosurfactant is Produced by *Pseudomonas aeruginosa*,” *Bionatura*, 8(4). Available at: https://doi.org/10.21931/RB/2023.08.04.25.
Andrä, J. *et al.* (2006) “Endotoxin-like properties of a rhamnolipid exotoxin from Burkholderia (*Pseudomonas*) plantarii: immune cell stimulation and biophysical characterization,” *Biological chemistry*, 387(3), pp. 301–310. Available at: https://doi.org/10.1515/BC.2006.040.

Angon, P.B. *et al.* (2024) “Sources, effects and present perspectives of heavy metals contamination: Soil, plants and human food chain,” *Heliyon*, 10(7), p. e28357. Available at: https://doi.org/10.1016/J.HELIYON.2024.E28357.

Ankulkar, R. *et al.* (2022) “Cytotoxicity of di-rhamnolipids produced by *Pseudomonas aeruginosa* RA5 against human cancerous cell lines,” *3 Biotech*, 12(11). Available at: https://doi.org/10.1007/S13205-022-03391-0.

Arshad, H. *et al.* (2020) “Evaluation of heavy metals in cosmetic products and their health risk assessment,” *Saudi Pharmaceutical Journal : SPJ*, 28(7), p. 779. Available at: https://doi.org/10.1016/J.JSPS.2020.05.006.

Berkat, S. *et al.* (2025) “Hydrocarbon biodegradation by strain BSP4 isolated from a wastewater treatment plant: insights into rhamnolipid production and characterization,” *International Journal of Environmental Science and Technology* [Preprint]. Available at: https://doi.org/10.1007/s13762-025-06413-5.

Buonocore, C. *et al.* (2023) “Evaluation of Antimicrobial Properties and Potential Applications of *Pseudomonas* gessardii M15 Rhamnolipids towards Multiresistant Staphylococcus aureus,” *Pharmaceutics*, 15(2), p. 700. Available at: https://doi.org/10.3390/PHARMACEUTICS15020700/S1.

Caiazza, N.C., Shanks, R.M.Q. and O’Toole, G.A. (2005) “Rhamnolipids modulate swarming motility patterns of *Pseudomonas aeruginosa*,” *Journal of bacteriology*, 187(21), pp. 7351–7361. Available at: https://doi.org/10.1128/JB.187.21.7351-7361.2005.

Cerqueira dos Santos, S. *et al.* (2024) “Production and characterization of rhamnolipids by *Pseudomonas aeruginosa* isolated in the Amazon region, and potential antiviral, antitumor, and antimicrobial activity,” *Scientific Reports*, 14(1), pp. 1–13. Available at: https://doi.org/10.1038/S41598-024-54828-W;SUBJMETA.

Chang, Q. *et al.* (2023) “The Synergistic Activity of Rhamnolipid Combined with Linezolid against Linezolid-Resistant Enterococcus faecium,” *Molecules (Basel, Switzerland)*, 28(22). Available at: https://doi.org/10.3390/MOLECULES28227630.

Chen, W. *et al.* (2020) “Enhanced biodegradation of crude oil by constructed bacterial consortium comprising salt-tolerant petroleum degraders and biosurfactant producers,” *International Biodeterioration & Biodegradation*, 154, p. 105047. Available at: https://doi.org/10.1016/J.IBIOD.2020.105047.

Chong, H. and Li, Q. (2017a) “Microbial production of rhamnolipids: Opportunities, challenges and strategies,” *Microbial Cell Factories*, 16(1), pp. 1–12. Available at: https://doi.org/10.1186/S12934-017-0753-2/FIGURES/3.

Chong, H. and Li, Q. (2017b) “Microbial production of rhamnolipids: Opportunities, challenges and strategies,” *Microbial Cell Factories*, 16(1), pp. 1–12. Available at: https://doi.org/10.1186/S12934-017-0753-2/FIGURES/3.

Christova, N. *et al.* (2013) “Chemical structure and in vitro antitumor activity of rhamnolipids from *Pseudomonas aeruginosa* BN10,” *Applied biochemistry and biotechnology*, 170(3), pp. 676–689. Available at: https://doi.org/10.1007/S12010-013-0225-Z.

CONAMA (2005) *Resolução357 DE 17/03/2005*. Available at: https://www.legisweb.com.br/legislacao/?id=102255 (Accessed: October 24, 2025).

Costa, J.A.V. *et al.* (2018) “Solid-State Fermentation for the Production of Biosurfactants and Their Applications,” *Current Developments in Biotechnology and Bioengineering: Current Advances in Solid-State Fermentation*, pp. 357–372. Available at: https://doi.org/10.1016/B978-0-444-63990-5.00016-5.

Dahrazma, B. and Mulligan, C.N. (2007) “Investigation of the removal of heavy metals from sediments using rhamnolipid in a continuous flow configuration,” *Chemosphere*, 69(5), pp. 705–711. Available at: https://doi.org/10.1016/J.CHEMOSPHERE.2007.05.037.

Davey, M.E., Caiazza, N.C. and O’Toole, G.A. (2003a) “Rhamnolipid Surfactant Production Affects Biofilm Architecture in *Pseudomonas aeruginosa* PAO1,” *Journal of Bacteriology*, 185(3), p. 1027. Available at: https://doi.org/10.1128/JB.185.3.1027-1036.2003.

Davey, M.E., Caiazza, N.C. and O’Toole, G.A. (2003b) “Rhamnolipid Surfactant Production Affects Biofilm Architecture in *Pseudomonas aeruginosa* PAO1,” *Journal of Bacteriology*, 185(3), p. 1027. Available at: https://doi.org/10.1128/JB.185.3.1027-1036.2003.

Davies, D.G. *et al.* (1998) “The involvement of cell-to-cell signals in the development of a bacterial biofilm,” *Science (New York, N.Y.)*, 280(5361), pp. 295–298. Available at: https://doi.org/10.1126/SCIENCE.280.5361.295.

Dobler, L. *et al.* (2016) “Rhamnolipids in perspective: gene regulatory pathways, metabolic engineering, production and technological forecasting,” *New Biotechnology*, 33(1), pp. 123–135. Available at: https://doi.org/10.1016/J.NBT.2015.09.005.

Donio, M.B.S. *et al.* (2013) “Halomonas sp. BS4, A biosurfactant producing halophilic bacterium isolated from solar salt works in India and their biomedical importance,” *SpringerPlus*, 2(1), p. 149. Available at: https://doi.org/10.1186/2193-1801-2-149.

Effendi, I. *et al.* (2017) “Detergent Disposal into Our Environmentand Its Impact on Marine Microbes,” *IOP Conference Series: Earth and Environmental Science*, 97(1). Available at: https://doi.org/10.1088/1755-1315/97/1/012030.

Eras-Muñoz, E. *et al.* (2022) “Microbial biosurfactants: a review of recent environmental applications,” *Bioengineered*, 13(5), pp. 12365–12391. Available at: https://doi.org/10.1080/21655979.2022.2074621,.

Fujita, K., Akino, T. and Yoshioka, H. (1988) “Characteristics of heat-stable extracellular hemolysin from *Pseudomonas aeruginosa*,” *Infection and immunity*, 56(5), pp. 1385–1387. Available at: https://doi.org/10.1128/IAI.56.5.1385-1387.1988.

Funston, S.J. *et al.* (2017) “Enhanced rhamnolipid production in Burkholderia thailandensis transposon knockout strains deficient in polyhydroxyalkanoate (PHA) synthesis,” *Applied Microbiology and Biotechnology*, 101(23), p. 8443. Available at: https://doi.org/10.1007/S00253-017-8540-X.

Gaur, S. *et al.* (2023) “Remediation of Waste Engine Oil Contaminated Soil using Rhamnolipid based Detergent Formulation,” *Materials Today: Proceedings*, 77, pp. 31–38. Available at: https://doi.org/10.1016/j.matpr.2022.08.452.

Gdaniec, B.G. *et al.* (2022) “*Pseudomonas aeruginosa* rhamnolipid micelles deliver toxic metabolites and antibiotics into Staphylococcus aureus,” *iScience*, 25(1), p. 103669. Available at: https://doi.org/10.1016/J.ISCI.2021.103669.

Glick, R. *et al.* (2010) “Increase in Rhamnolipid Synthesis under Iron-Limiting Conditions Influences Surface Motility and Biofilm Formation in *Pseudomonas aeruginosa*,” *Journal of Bacteriology*, 192(12), p. 2973. Available at: https://doi.org/10.1128/JB.01601-09.

Haba, E. *et al.* (2014) “Complex rhamnolipid mixture characterization and its influence on DPPC bilayer organization,” *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1838(3), pp. 776–783. Available at: https://doi.org/10.1016/J.BBAMEM.2013.11.004.

Häußbler, S. *et al.* (2003) “Structural and Functional Cellular Changes Induced by Burkholderia pseudomallei Rhamnolipid,” *Infection and Immunity*, 71(5), p. 2970. Available at: https://doi.org/10.1128/IAI.71.5.2970-2975.2003.

Henkel, M. *et al.* (2012) “Rhamnolipids as biosurfactants from renewable resources: Concepts for next-generation rhamnolipid production,” *Process Biochemistry*, 47(8), pp. 1207–1219. Available at: https://doi.org/10.1016/J.PROCBIO.2012.04.018.

Hörmann, B. *et al.* (2010) “Rhamnolipid production by Burkholderia plantarii DSM 9509T,” *European Journal of Lipid Science and Technology*, 112(6), pp. 674–680. Available at: https://doi.org/10.1002/EJLT.201000030.

Hošková, M. *et al.* (2015) “Structural and physiochemical characterization of rhamnolipids produced by Acinetobacter calcoaceticus, Enterobacter asburiae and *Pseudomonas aeruginosa* in single strain and mixed cultures,” *Journal of Biotechnology*, 193, pp. 45–51. Available at: https://doi.org/10.1016/J.JBIOTEC.2014.11.014.

Hosseini, S., Sharifi, R. and Habibi, A. (2025) “Efficient bioremediation of crude oil contaminated soil by a consortium of in-situ biosurfactant producing hydrocarbon-degraders,” *Scientific Reports*, 15(1). Available at: https://doi.org/10.1038/s41598-025-05035-8.

Hsu, C.Y. *et al.* (2025) “Biosurfactants: Properties, applications and emerging trends,” *South African Journal of Chemical Engineering*, 53, pp. 21–39. Available at: https://doi.org/10.1016/J.SAJCE.2025.04.002.

Jiang, L. *et al.* (2014) “Rhamnolipids elicit the same cytotoxic sensitivity between cancer cell and normal cell by reducing surface tension of culture medium,” *Applied microbiology and biotechnology*, 98(24), pp. 10187–10196. Available at: https://doi.org/10.1007/S00253-014-6065-0.

Johnson, P. *et al.* (2021) “Effect of synthetic surfactants on the environment and the potential for substitution by biosurfactants,” *Advances in Colloid and Interface Science*, 288, p. 102340. Available at: https://doi.org/10.1016/J.CIS.2020.102340.

Kaczorek, E. *et al.* (2018) “The Impact of Biosurfactants on Microbial Cell Properties Leading to Hydrocarbon Bioavailability Increase,” *Colloids and Interfaces 2018, Vol. 2, Page 35*, 2(3), p. 35. Available at: https://doi.org/10.3390/COLLOIDS2030035.

Khiyavi, A.D. *et al.* (2020) “Synergistic effect of rhamnolipid and saponin biosurfactants on removal of heavy metals from oil contaminated soils,” *Tenside, Surfactants, Detergents*, 57(2), pp. 109–114. Available at: https://doi.org/10.3139/113.110672.

Kou, B. *et al.* (2024) “Effect of soil organic matter-mediated electron transfer on heavy metal remediation: Current status and perspectives,” *Science of The Total Environment*, 917, p. 170451. Available at: https://doi.org/10.1016/J.SCITOTENV.2024.170451.

Kownatzki, R., Tümmler, B. and Döring, G. (1987) “Rhamnolipid of *Pseudomonas aeruginosa* in sputum of cystic fibrosis patients,” *Lancet (London, England)*, 1(8540), pp. 1026–1027. Available at: https://doi.org/10.1016/S0140-6736(87)92286-0.

Kumar, R. *et al.* (2023) “Sustainable rhamnolipids production in the next decade – Advancing with Burkholderia thailandensis as a potent biocatalytic strain,” *Microbiological Research*, 272, p. 127386. Available at: https://doi.org/10.1016/J.MICRES.2023.127386.

Lima, T.M.S. *et al.* (2011) “Simultaneous phenanthrene and cadmium removal from contaminated soil by a ligand/biosurfactant solution,” *Biodegradation 2011 22:5*, 22(5), pp. 1007–1015. Available at: https://doi.org/10.1007/S10532-011-9459-Z.

Liu, G. *et al.* (2018) “Advances in applications of rhamnolipids biosurfactant in environmental remediation: A review,” *Biotechnology and Bioengineering*, 115(4), pp. 796–814. Available at: https://doi.org/10.1002/bit.26517.

Liu, H.Y., Prentice, E.L. and Webber, M.A. (2024) “Mechanisms of antimicrobial resistance in biofilms,” *npj Antimicrobials and Resistance*, 2(1), pp. 1–10. Available at: https://doi.org/10.1038/S44259-024-00046-3;SUBJMETA.

Liu, Q. *et al.* (2015) “Production of surfactin isoforms by Bacillus subtilis BS-37 and its applicability to enhanced oil recovery under laboratory conditions,” *Biochemical Engineering Journal*, 93, pp. 31–37. Available at: https://doi.org/10.1016/J.BEJ.2014.08.023.

Lu, L. *et al.* (2023) “Rhamnolipid Biosurfactants Enhance Microbial Oil Biodegradation in Surface Seawater from the North Sea,” *ACS ES&T Water*, 3(8), pp. 2255–2266. Available at: https://doi.org/10.1021/ACSESTWATER.3C00048.

Lu, Y. *et al.* (2018) “Strategies to improve micelle stability for drug delivery,” *Nano research*, 11(10), p. 4985. Available at: https://doi.org/10.1007/S12274-018-2152-3.

Luketich, M., Samouei, H. and Nasrabadi, H. (2024) “A novel approach to produced water management using surfactants for water-wise energy production,” *Geoenergy Science and Engineering*, 242, p. 213187. Available at: https://doi.org/10.1016/J.GEOEN.2024.213187.

Luo, X. *et al.* (2025) “Integrated thermal and biostimulant enhanced bioremediation of PAHs and arsenic co-contaminated soil by an indigenous microbial consortium: Performance, mechanisms and limitations,” *Environmental Pollution*, 377. Available at: https://doi.org/10.1016/j.envpol.2025.126365.

Malakar, C. *et al.* (2024) “Antibiofilm and wound healing efficacy of rhamnolipid biosurfactant against pathogenic bacterium Staphylococcus aureus,” *Microbial Pathogenesis*, 195. Available at: https://doi.org/10.1016/j.micpath.2024.106855.

McClure, C.D. and Schiller, N.L. (1992) “Effects of *Pseudomonas aeruginosa* rhamnolipids on human monocyte-derived macrophages,” *Journal of leukocyte biology*, 51(2), pp. 97–102. Available at: https://doi.org/10.1002/JLB.51.2.97.

Millioli, V.S. *et al.* (2009) *BIOREMEDIATION OF CRUDE OIL-BEARING SOIL: EVALUATING THE EFFECT OF RHAMNOLIPID ADDITION TO SOIL TOXICITY AND TO CRUDE OIL BIODEGRADATION EFFICIENCY*, *Global NEST Journal*.

Mulligan, C.N. (2005) “Environmental applications for biosurfactants,” *Environmental Pollution*, 133(2), pp. 183–198. Available at: https://doi.org/10.1016/J.ENVPOL.2004.06.009.

Mulligan, C.N.., Mudhoo, Ackmez. and Sharma, S.K.. (2014) “Biosurfactants : research trends and applications.”

Muthaliar, A., Sevakumaran, V. and Bhubalan, K. (2019) *Production and toxicity evaluation of rhamnolipids produced byPseudomonas strain on L6 and HepG2 cells*. Available at:

https://www.researchgate.net/publication/343472335_PRODUCTION_AND_TOXICITY_EVALUATION_OF_RHAMNOLIPIDS_PRODUCED_BY_*Pseudomonas*_STRAINS_ON_L6_AND_HepG2_CELLS (Accessed: October 16, 2025).

Nicolò, M.S. *et al.* (2017) “Carbon source effects on the mono/dirhamnolipid ratio produced by *Pseudomonas aeruginosa* L05, a new human respiratory isolate,” *New Biotechnology*, 39(Pt A), pp. 36–41. Available at: https://doi.org/10.1016/j.nbt.2017.05.013.

Pamp, S.J. and Tolker-Nielsen, T. (2007) “Multiple roles of biosurfactants in structural biofilm development by *Pseudomonas aeruginosa*,” *Journal of bacteriology*, 189(6), pp. 2531–2539. Available at: https://doi.org/10.1128/JB.01515-06.

Pang, A.P. *et al.* (2024) “Highly efficient production of rhamnolipid in P. putida using a novel sacB-based system and mixed carbon source,” *Bioresource Technology*, 394, p. 130220. Available at: https://doi.org/10.1016/J.BIORTECH.2023.130220.

Potapov, K. *et al.* (2023) “Effects of Natural Rhamnolipid Mixture on Dioleoylphosphatidylcholine Model Membrane Depending on Method of Preparation and Sterol Content,” *Membranes*, 13(1), p. 112. Available at: https://doi.org/10.3390/MEMBRANES13010112/S1.

Purwasena, I.A., Astuti, D.I. and Utami, S.G. (2020) “Nitrogen optimization on rhamnolipid biosurfactant production from pseudoxanthomonas sp. G3 and its preservation techniques,” *Sains Malaysiana*, 49(9), pp. 2119–2127. Available at: https://doi.org/10.17576/JSM-2020-4909-10.

Quan, K. *et al.* (2022) “Water in bacterial biofilms: pores and channels, storage and transport functions,” *Critical reviews in microbiology*, 48(3), pp. 283–302. Available at: https://doi.org/10.1080/1040841X.2021.1962802.

Rahim, R. *et al.* (2001) “Cloning and functional characterization of the *Pseudomonas aeruginosa* rhlC gene that encodes rhamnosyltransferase 2, an enzyme responsible for di-rhamnolipid biosynthesis,” *Molecular microbiology*, 40(3), pp. 708–718. Available at: https://doi.org/10.1046/J.1365-2958.2001.02420.X.

Rahimi, K. *et al.* (2019) “Cytotoxic effects of mono- and di-rhamnolipids from *Pseudomonas aeruginosa* MR01 on MCF-7 human breast cancer cells,” *Colloids and Surfaces B: Biointerfaces*, 181, pp. 943–952. Available at: https://doi.org/10.1016/J.COLSURFB.2019.06.058.

Rather, M.A. *et al.* (2025) “Biodegradation of crude oil by biosurfactant-producing microaerophilic bacterium *Pseudomonas aeruginosa* MAR1,” *Science of the Total Environment*, 991. Available at: https://doi.org/10.1016/j.scitotenv.2025.179876.

Reynolds, D. and Kollef, M. (2021) “The Epidemiology and Pathogenesis and Treatment of *Pseudomonas aeruginosa* Infections: An Update,” *Drugs*, 81(18), p. 2117. Available at: https://doi.org/10.1007/S40265-021-01635-6.

Rojo, F. (2010) “Carbon catabolite repression in *Pseudomonas*: optimizing metabolic versatility and interactions with the environment,” *FEMS microbiology reviews*, 34(5), pp. 658–684. Available at: https://doi.org/10.1111/J.1574-6976.2010.00218.X.

Saadati, F. *et al.* (2022) “Effect of MA01 rhamnolipid on cell viability and expression of quorum-sensing (QS) genes involved in biofilm formation by methicillin-resistant Staphylococcus aureus,” *Scientific Reports*, 12(1), pp. 1–12. Available at: https://doi.org/10.1038/S41598-022-19103-W;SUBJMETA.

Safari, P. *et al.* (2023) “Evaluation of surface activity of rhamnolipid biosurfactants produced from rice bran oil through dynamic surface tension,” *Journal of Petroleum Exploration and Production Technology*, 13(10), pp. 2139–2153. Available at: https://doi.org/10.1007/S13202-023-01660-Z/FIGURES/1.

Sarubbo, L.A. *et al.* (2022) “Biosurfactants: Production, properties, applications, trends, and general perspectives,” *Biochemical Engineering Journal*, 181, p. 108377. Available at: https://doi.org/10.1016/J.BEJ.2022.108377.

Schellenberger, R. *et al.* (2021) “Bacterial rhamnolipids and their 3-hydroxyalkanoate precursors activate Arabidopsis innate immunity through two independent mechanisms,” *Proceedings of the National Academy of Sciences of the United States of America*, 118(39), p. e2101366118. Available at: https://doi.org/10.1073/PNAS.2101366118/-/DCSUPPLEMENTAL.

Semkova, S. *et al.* (2021) “Rhamnolipid Biosurfactants—Possible Natural Anticancer Agents and Autophagy Inhibitors,” *Separations 2021, Vol. 8, Page 92*, 8(7), p. 92. Available at: https://doi.org/10.3390/SEPARATIONS8070092.

Sen, S. *et al.* (2020) “Rhamnolipid exhibits anti-biofilm activity against the dermatophytic fungi Trichophyton rubrum and Trichophyton mentagrophytes,” *Biotechnology Reports*, 27, p. e00516. Available at: https://doi.org/10.1016/J.BTRE.2020.E00516.

Shao, B. *et al.* (2017) “Effects of rhamnolipids on microorganism characteristics and applications in composting: A review,” *Microbiological Research*, 200, pp. 33–44. Available at: https://doi.org/10.1016/J.MICRES.2017.04.005.

Sharaf, M. *et al.* (2022) “Rhamnolipid-Coated Iron Oxide Nanoparticles as a Novel Multitarget Candidate against Major Foodborne E. coli Serotypes and Methicillin-Resistant S. aureus,” *Microbiology Spectrum*, 10(4), pp. e00250-22. Available at: https://doi.org/10.1128/SPECTRUM.00250-22.

Sonmez, V.Z., Akarsu, C. and Sivri, N. (2025) “Rhamnolipid: nature-based solution for the removal of microplastics from the aquatic environment,” *Integrated environmental assessment and management*, 21(2), pp. 350–359. Available at: https://doi.org/10.1093/INTEAM/VJAE037.

Sorour, A. *et al.* (2025) “Rhamnolipid from *Pseudomonas* sp. as a green surfactant for enhanced phytoremediation,” *Scientific reports*, 15(1), p. 29780. Available at: https://doi.org/10.1038/S41598-025-14244-0;KWRD.

Tan, Y.N. and Li, Q. (2018) “Microbial production of rhamnolipids using sugars as carbon sources,” *Microbial Cell Factories*, 17(1), p. 89. Available at: https://doi.org/10.1186/S12934-018-0938-3.

Thanomsub, B. *et al.* (2006) “Chemical structures and biological activities of rhamnolipids produced by *Pseudomonas aeruginosa* B189 isolated from milk factory waste,” *Bioresource Technology*, 97(18), pp. 2457–2461. Available at: https://doi.org/10.1016/j.biortech.2005.10.029.

Touabi, L. *et al.* (2025) “Antiviral Activity of Rhamnolipids Nano-Micelles Against Rhinoviruses—In Silico Docking, Molecular Dynamic Analysis and In-Vitro Studies,” *Current Issues in Molecular Biology*, 47(5), p. 333. Available at: https://doi.org/10.3390/CIMB47050333/S1.

Tripathi, L. *et al.* (2019) “Biosynthesis of rhamnolipid by a Marinobacter species expands the paradigm of biosurfactant synthesis to a new genus of the marine microflora,” *Microbial Cell Factories*, 18(1), pp. 1–12. Available at: https://doi.org/10.1186/S12934-019-1216-8/FIGURES/4.

Wu, B. *et al.* (2022a) “Biosurfactant production by Bacillus subtilis SL and its potential for enhanced oil recovery in low permeability reservoirs,” *Scientific Reports*, 12(1), pp. 1–10. Available at: https://doi.org/10.1038/S41598-022-12025-7;SUBJMETA.
Wu, B. *et al.* (2022b) “Research advances of microbial enhanced oil recovery,” *Heliyon*, 8(11), p. e11424. Available at: https://doi.org/10.1016/J.HELIYON.2022.E11424.

Wuana, R.A. *et al.* (2011) “Heavy Metals in Contaminated Soils: A Review of Sources, Chemistry, Risks and Best Available Strategies for Remediation,” *International Scholarly Research Notices*, 2011(1), p. 402647. Available at: https://doi.org/10.5402/2011/402647.

Xu, Q. *et al.* (2024) “Cytotoxic rhamnolipid micelles drive acute virulence in *Pseudomonas aeruginosa*,” *Infection and Immunity*, 92(3), pp. e00407-23. Available at: https://doi.org/10.1128/IAI.00407-23.

Yang, X. *et al.* (2020) “Sub-CMC solubilization of n-alkanes by rhamnolipid biosurfactant: the Influence of rhamnolipid molecular structure,” *Colloids and Surfaces B: Biointerfaces*, 192, p. 111049. Available at: https://doi.org/10.1016/J.COLSURFB.2020.111049.

Zhao, F. *et al.* (2022) “Comparative study on antimicrobial activity of mono-rhamnolipid and di-rhamnolipid and exploration of cost-effective antimicrobial agents for agricultural applications,” *Microbial Cell Factories*, 21(1), pp. 1–10. Available at: https://doi.org/10.1186/S12934-022-01950-X/TABLES/4.

Zhong, H. *et al.* (2016) “Effect of low-concentration rhamnolipid on transport of *Pseudomonas aeruginosa* ATCC 9027 in an ideal porous medium with hydrophilic or hydrophobic surfaces,” *Colloids and Surfaces B: Biointerfaces*, 139, pp. 244–248. Available at: https://doi.org/10.1016/J.COLSURFB.2015.11.024.

Zhou, Xihe *et al.* (2023) “Environmental and human health impacts of volatile organic compounds: A perspective review,” *Chemosphere*, 313, p. 137489. Available at: https://doi.org/10.1016/J.CHEMOSPHERE.2022.137489.

Zhu, P. *et al.* (2022) “Rhamnolipids from non-pathogenic Acinetobacter calcoaceticus: Bioreactor-scale production, characterization and wound healing potency,” *New Biotechnology*, 67, pp. 23–31. Available at: https://doi.org/10.1016/J.NBT.2021.12.001.

CHAPTER II - BIOTECHNOLOGICAL PROSPECTS OF RHAMNOLIPIDS: PRODUCTION, SURFACE TENSION ACTIVITY, ENVIRONMENTAL APPLICATIONS AND ANTIMICROBIAL POTENTIAL.

Abstract

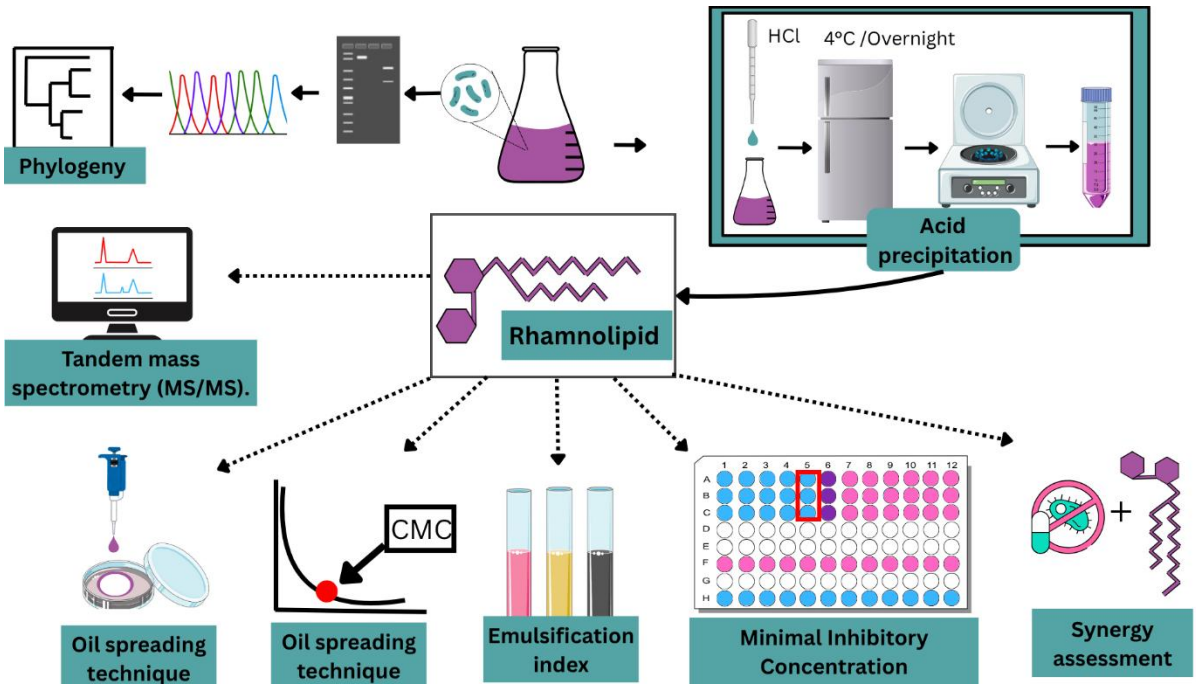
Rhamnolipids are microbial biosurfactants with promising applications in environmental, biomedical, and agricultural fields due to their ability to reduce surface tension, as well as their emulsifying and antimicrobial properties. In this study, three *Pseudomonas aeruginosa* isolates (LBBMA 58, LBBMA 79, and LBBMA 92) were cultivated under controlled conditions to evaluate rhamnolipid production and characterize the congeners produced by each strain. Crude extracts were recovered, purified, and characterized using several methodologies, including the oil spreading technique, determination of the critical micelle concentration (CMC), emulsification index (E24), and LC–MS/MS congener profiling.

In addition, antimicrobial assays were conducted against *Escherichia coli* ECC, *Staphylococcus aureus* SAC, and the phytopathogenic fungus *Corynespora cassiicola*. For *E. coli* ECC and *S. aureus* SAC, synergy assays were performed to

evaluate the behavior of rhamnolipids in combination with chloramphenicol. Surface tension measurements revealed that all extracts were able to reduce the surface tension of water to values below 30 mN m^{-1}. LC–MS/MS analysis confirmed the presence of four rhamnolipid congeners, predominantly di-rhamnolipids (Rha–Rha–C10–C10).

Rhamnolipids produced by *P. aeruginosa* LBBMA 58 exhibited the strongest inhibitory effect against both bacterial species. However, rhamnolipids showed antagonistic effects when combined with chloramphenicol. Unexpectedly, the tested concentrations stimulated fungal growth rather than inhibiting it. Overall, this study demonstrates that species- and strain-dependent rhamnolipid composition strongly influences antimicrobial activity. These findings highlight the need for careful evaluation of rhamnolipid–antibiotic interactions and species-specific effects prior to their biomedical or agricultural application.

Graphical abstract



Graphical abstract. Source: authors

Keywords: biosurfactants; isolates; antimicrobial; characterization; antagonist.

2.1 Introduction

Surfactants, or surface-active agents, are amphiphilic compounds that reduce the surface tension of water and modify interfacial properties, enabling diverse applications in medicine, agriculture, and environmental science. Their functionality stems from a molecular structure comprising a hydrophilic head and a hydrophobic tail. This unique characteristic allows surfactants to interact efficiently with both nonpolar and aqueous compounds. Owing to this dual affinity, rhamnolipids are able to stabilize emulsions, form micelles, and, in many cases, solubilize hydrophobic substances (Alwadani and Fatehi, 2018; Yang et al., 2020). With the capacity to reduce surface tension to values as low as 26 mN m^{-1}, rhamnolipids stand out among other common biosurfactants (Mulligan, 2005; Liu et al., 2015; Safari et al., 2023).

Valued for their cost-effectiveness, synthetic surfactants are extensively used in diverse sectors such as agriculture, petroleum recovery, cosmetics, and detergent production (Hsu et al., 2025). This widespread demand has resulted in an annual global production exceeding 15 million tons. Their large-scale use was largely driven by economic advantages, often preceding a full understanding of their long-term environmental and health impacts. Subsequent research demonstrated that the extensive use of synthetic surfactants has caused significant environmental damage and raised concerns regarding human health. The low biodegradability and toxicity of many synthetic surfactants toward aquatic organisms have led to critical environmental disturbances, including alterations in oxygen availability and aquatic ecosystem dynamics. Moreover, the degradation of some surfactants releases harmful by-products, such as alkylphenols, which further exacerbate environmental damage (Ying, 2006; Effendi et al., 2017; Johnson et al., 2021; Luketich, Samouei and Nasrabadi, 2024). Consequently, regulatory agencies have imposed restrictions on their use, such as Brazilian legislation that limits anionic surfactant concentrations in freshwater systems to 0.5 mg L^{-1} (CONAMA, 2005).

In contrast, biosurfactants are produced by microorganisms and plants and have gained increasing attention due to their biodegradability, low toxicity, and environmental compatibility (Sarubbo et al., 2022). Although biosurfactants are structurally diverse, they are commonly classified into high-molecular-weight (HMW) and low-molecular-weight (LMW) groups, which broadly reflect their primary functions. HMW biosurfactants, including polysaccharides and lipoproteins, mainly act as bioemulsifiers, whereas LMW biosurfactants, such as glycolipids and lipopeptides, are more effective at reducing surface and interfacial tensions (Johnson et al., 2021). Among glycolipids, rhamnolipids and sophorolipids are the most extensively studied; however, the superior surface-active properties of rhamnolipids position them as the most promising LMW biosurfactants (Mulligan, Mudhoo and Sharma, 2014).

Rhamnolipids are characterized by the presence of one or two rhamnose sugar units linked to one or two β-hydroxy fatty acid chains. Variations in chain length, degree of unsaturation, and the number of rhamnose and β-hydroxy fatty acid moieties generate considerable structural diversity, which in turn influences their physicochemical and biological properties (Abdel-Mawgoud, Lépine and Déziel, 2010; Hošková et al., 2015). To date, more than sixty rhamnolipid congeners have been identified, with di-rhamnolipids such as Rha–Rha–C10–C10 frequently reported as predominant. In recent years, rhamnolipid-producing microorganisms have been identified across several genera, including Burkholderia, Acinetobacter, Enterobacter, and Pseudomonas (Hörmann et al., 2010; Hošková et al., 2015; Araujo et al., 2020; Zhu et al., 2022). This taxonomic diversity is reflected in the wide structural variability of rhamnolipids, which is closely associated with differential gene expression. In Pseudomonas, for example, rhamnolipid biosynthesis and congener diversity are primarily regulated by the expression of the rhlA, rhlB, and rhlC genes. The regulation of these genes is strongly influenced by environmental conditions and quorum sensing networks, as well as by resource availability (Makkar, Cameotra and Banat, 2011; Schmidberger et al., 2013; Bahia et al., 2018).

Beyond their physicochemical properties and classification as secondary metabolites, rhamnolipids have evolved to play fundamental ecological and physiological roles that enhance the survival and competitiveness of producing strains. Numerous studies have demonstrated that rhamnolipid synthesis is essential for facilitating both swarming and sliding motility in bacteria. In addition, these biosurfactants are key

components in biofilm formation, maintenance, and structural organization. Within complex microbial communities, rhamnolipids can act as antimicrobial and antibiofilm agents, thereby significantly contributing to the ecological fitness of the producing organisms (Davey, Caiazza and O'Toole, 2003; Caiazza, Shanks and O'Toole, 2005; Pamp and Tolker-Nielsen, 2007).

Rhamnolipids are therefore highly attractive for a wide range of industrial applications, including bioremediation, wastewater treatment, enhanced oil recovery, agriculture, and pharmaceutical formulations (Liu et al., 2018; Ambaye et al., 2022; Wu et al., 2022; Sonmez, Akarsu and Sivri, 2025). This versatility arises from their strong surface- and interfacial-tension-reducing capacity, structural diversity, and multifunctional behavior. Nevertheless, strain-dependent differences in productivity, biological activity, and congener distribution make detailed characterization essential to ensure consistent performance and appropriate application.

In this context, the present study investigated rhamnolipid production by three Pseudomonas aeruginosa strains (LBBMA 58, LBBMA 79, and LBBMA 92) and evaluated selected physicochemical properties and antimicrobial potential. By combining biochemical assays with LC–MS/MS characterization, this work aimed to elucidate strain-dependent variability in rhamnolipid performance. Understanding these differences is essential not only for advancing fundamental knowledge of rhamnolipids but also for guiding their effective application across diverse fields.

2.2 Methodology

2.2.1 Identification of the Pseudomonas isolates through multilocus sequence analysis (MLSA)

The experiment focused on three bacterial isolates previously characterized as biosurfactant producers: *Pseudomonas* sp. LBBMA 58, *Pseudomonas* sp. LBBMA 79, and *Pseudomonas* sp. LBBMA 92. These strains were obtained from the culture collection of the Laboratório de Biotecnologia e Biodiversidade para o Meio Ambiente (LBBMA) at the Universidade Federal de Viçosa (UFV), Brazil.

Genomic DNA was extracted from each bacterial strain using the BioSpin Bacteria Genomic DNA Extraction Kit (BioFlux, China), following the manufacturer's instructions. DNA quality and integrity were assessed by agarose gel electrophoresis.

In addition, DNA concentration was quantified using a Qubit 2.0 fluorometer (Life Technologies, USA).

For molecular identification, a multilocus sequence analysis (MLSA) approach was employed. The target genes selected for amplification and sequencing were the 16S rRNA gene, DNA gyrase subunit B (*gyrB*), and RNA polymerase sigma factor (*rpoD*). The 16S rRNA gene is widely used for bacterial identification due to its universal presence and conserved regions (Clarridge, 2004). The inclusion of *rpoD* and *gyrB* was strategic, as these housekeeping genes exhibit appropriate evolutionary rates and have been extensively used to resolve phylogenetic relationships among closely related *Pseudomonas* species (Yang Liu et al., 2017).

PCR amplification of the 16S rRNA, *rpoD*, and *gyrB* genes was carried out using their respective primer sets (Table 1). PCR amplification of the *rpoD*, *gyrB*, and 16S rRNA genes was performed using gene-specific primer pairs for each target gene. The sequences of all primers and the corresponding PCR amplification parameters are detailed in Table 1.

Table 1. Target genes and primer sequences

Gene	Primer's name	Primer's sequence
16S rRNA	27F	(5′-GAGTTTGATCATGGCTCAG-3′)
	1492R	5′-GGTTACCTTGTTACGACTT-3′
rpoD	*rpoD*-F	5′-ATGTCCGGAAAAGCGCAACAGCARTCTCG-3′
	rpoD-R	5′-CGGTTGGTGTACTTYTTGGCGAT-3′
gyrB	*gyrB*-F	5′-GGTGGTCGATAACTCCATCG-3′

	gyrB-R	5′-CGCTGAGGAATGTTGTTGGT-3′

Expected amplicon sizes were approximately 1,500 bp for the 16S rRNA gene, 760 bp for *rpoD*, and 1,200 bp for *gyrB* (Yamamoto & Harayama, 1995; Savli et al., 2003; Janda & Abbott, 2007).

Amplicons were sequenced in both directions, assembled, aligned, and manually inspected to generate high-quality consensus sequences using DNA Baser Assembler software. The resulting sequences were subsequently subjected to BLAST nucleotide (BLASTn) searches against the NCBI database to identify the closest related species. Based on BLAST results, reference sequences of the 16S rRNA, *rpoD*, and *gyrB* genes from related taxa were retrieved from GenBank.

Cellvibrio japonicus ADPT3-ISOMALTOSE was selected as the outgroup. After reference selection, sequences corresponding to the three genes for each species were concatenated using the software Mesquite v4.01 (https://www.mesquiteproject.org/). The concatenated sequences were then realigned using MAFFT and MEGA v12. Phylogenetic analyses were performed using the Maximum Likelihood method. Finally, phylogenetic tree visualization was generated using the Interactive Tree of Life (iTOL) platform (https://itol.embl.de).

2.2.2 Production of biosurfactants

The selected bacterial strains were reactivated on TSA (Tryptic Soy Agar) medium for 24–48 h at 30 °C. Subsequently, a single colony from each strain was transferred to TSB (Tryptic Soy Broth) medium and incubated at 30 °C. After 72 h of incubation, the optical density (OD) was measured using a spectrophotometer. The resulting cultures were used as inocula to inoculate fresh culture medium at an initial optical density (OD_{600}) of 0.1.

For each replicate, the Pseudomonas strain was cultivated in six 250 mL Erlenmeyer flasks, each containing 150 mL of mineral medium. The contents of the six flasks were then combined to form a single replicate. The mineral medium was composed of (g

L^{-1}): K_2HPO_4, 13.9; KH_2PO_4, 2.7; $NaNO_3$, 4.24; $C_6H_{12}O_2$ (glucose), 20; and yeast extract, 0.05, and was supplemented with 50 mL of a micronutrient solution. The micronutrient solution consisted of (g L^{-1}): EDTA, 0.5; $MgSO_4 \cdot 7H_2O$, 3.0; $MnSO_4 \cdot 4H_2O$, 0.5; NaCl, 1.0; $CaCl_2 \cdot 2H_2O$, 0.1; $CaCl_2 \cdot 6H_2O$, 0.1; $ZnSO_4 \cdot 7H_2O$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.1; $CuSO_4 \cdot 5H_2O$, 0.01; $Na_2MoO_4 \cdot 2H_2O$, 0.01; Na_2O_4Se, 0.01; $NaWO_4 \cdot 2H_2O$, 0.01; and $NiCl_2 \cdot 6H_2O$, 0.02. The flasks were incubated for 72 h at 35 °C under agitation at 180 rpm.

The mineral medium was selected because it provides a chemically defined and reproducible composition, allowing better control of nutrient availability and minimizing variability associated with complex media. This type of medium is commonly used for the cultivation of *Pseudomonas* spp. and for studies focused on the production of extracellular metabolites. The working volume (150 mL) in 250 mL Erlenmeyer flasks was chosen to maintain an appropriate headspace-to-liquid ratio, ensuring adequate oxygen transfer during orbital shaking at 180 rpm.

2.2.3 Recovery of the biosurfactants

To obtain the biosurfactants, a modified version of the methodology described by Araujo et al. (2020) was employed. The cultures were centrifuged at 12,000 × g for 15 min at 4 °C. The pH of the resulting cell-free supernatants was adjusted to 2–3 using HCl (1 mol L^{-1}), and the samples were stored overnight at 4 °C to allow biosurfactant precipitation. Subsequently, a second centrifugation step was performed under the same conditions.

The resulting precipitate was resuspended in 20 mL of ultrapure water, homogenized using a vortex mixer, and centrifuged at 10,000 × g for 15 min at 4 °C. This washing step was repeated once to further purify the biosurfactant fraction. After the final centrifugation, NaOH was added to adjust the pH to values above 6.

The precipitate was then resuspended in 5 mL of ethanol, filtered through a 0.22 µm membrane, and dried using a rotary vacuum concentrator (RVC 2-33 CD plus, CHRIST) at 35 °C. Once completely dried, the biosurfactant mass was determined using a precision analytical balance.

2.2.4 Characterization and quantification of biosurfactants

To characterize the chemical structures of the biosurfactants and quantify the individual congeners present in each extract, the previously dried material was

resuspended in ethanol to obtain a final concentration of 500 mg mL^{-1} and subsequently sent to the Biomolecule Analysis Core Facility (NuBioMol, UFV) for analysis. A commercial rhamnolipid standard was included as a reference control and processed under the same conditions as the samples of interest.

Aliquots of 5 µL were injected into an LC–MS/MS system consisting of an Agilent 1200 Infinity Series liquid chromatograph coupled to a triple quadrupole mass spectrometer (6430, Agilent Technologies). Chromatographic separation of rhamnolipid congeners was achieved using a ZORBAX RRHT StableBond CN column (3.0 × 150 mm, 1.8 µm; Agilent), equipped with a ZORBAX SB-CN guard column (1.8 µm; Agilent).

The mobile phase consisted of two solvents: (A) 0.02% acetic acid in water and (B) 0.02% acetic acid in acetonitrile. Gradient elution was performed according to the following program (%B over time, min): 0/50; 6/70; 10/100; 16/100; 18/55; and 20/55. The flow rate was set to 0.25 mL min^{-1}, and the column temperature was maintained at 23 °C.

Mass spectrometric detection was carried out using electrospray ionization (ESI) under the following conditions: gas temperature of 300 °C, nitrogen flow rate of 10 L min^{-1}, nebulizer pressure of 35 psi, and capillary voltage of 4,000 V.

Finally, the mass spectral data obtained were compared with previously reported literature values to identify and assign each rhamnolipid congener detected in the samples.

2.2.5 Oil spreading technique

The oil spreading technique was performed following a modified version of the method described by Youssef et al. (2004). Briefly, a Petri dish (150 × 20 mm) was filled with 70 mL of distilled water, after which a single drop of crude oil was carefully added to the water surface. Once a uniform oil film was formed, 10 µL of cell-free culture supernatant was gently deposited onto the center of the oil layer. After 30 s, biosurfactant activity was assessed by measuring the diameter of the clear halo formed as a result of oil displacement. Culture medium without bacterial growth was used as a negative control (blank).

This procedure was repeated after the biosurfactant purification process. In this case, the dried biosurfactant extract was resuspended in distilled water to obtain a final concentration of 1,000 mg mL^{-1}, and the resulting solution was evaluated using the oil

spreading assay. Biosurfactant activity was compared with a negative control consisting of distilled water, and halo diameters were measured in triplicate for each rhamnolipid sample.

2.2.6 Determination of Critical Micelle Concentration

Twenty milliliters of the biosurfactant solution (1,000 mg mL^{-1}) were transferred to a Petri dish for the initial surface tension measurement. From this solution, a series of serial dilutions was prepared, reducing the concentration by 25% at each step. This procedure was repeated successively until a final concentration of 0.18 mg mL^{-1} was reached. After each dilution, the sample was immediately analyzed using a tensiometer (KRÜSS DSA-T 11 EC) to determine surface tension values.

To determine the critical micelle concentration (CMC), surface tension measurements were plotted against biosurfactant concentration, and the CMC was defined as the concentration at which the curve reached a plateau, indicating that further increases in biosurfactant concentration no longer resulted in a significant decrease in surface tension (Perinelli et al., 2020).

2.2.7 Determination of emulsification index (E24)

The emulsifying activity of each biosurfactant extract was evaluated using a modified version of the method described by Cerqueira dos Santos et al. (2024). Briefly, 4 mL of the biosurfactant aqueous solution (diluted in distilled water) was added to test tubes containing an equal volume (4 mL) of the oil phase to be tested in diesel, petroleum, or soybean oil. The biosurfactant concentration for each microorganism was adjusted according to its respective critical micelle concentration (CMC), ensuring that all biosurfactant solutions were tested at 1× CMC.

The tubes were sealed and homogenized using a vortex mixer at high speed for 2 min and subsequently left undisturbed at room temperature for 24 h. All assays were performed in triplicate for each biological sample. The emulsification index (E_{24}) was then calculated according to Equation (1).

$$(1) \qquad Emulsification\ Index\ (E_{24}) = \left(\frac{Height\ of\ the\ emulsion\ layer}{Total\ height\ of\ the\ liquid\ column}\right) \times 100$$

2.2.8 Minimum inhibitory concentration (MIC) of biosurfactants against bacteria

2.2.8.1 Biosurfactant MIC value

The antimicrobial activity of the biosurfactants was evaluated by assessing their ability to inhibit the growth of two pathogenic bacteria: the Gram-negative *Escherichia coli* (ECC) and the Gram-positive *Staphylococcus aureus* (SAC). Both isolates were obtained from goats diagnosed with bacterial mastitis. The strains were provided by the Department of Veterinary Medicine at the Universidade Federal de Viçosa (UFV), Minas Gerais, Brazil, and are currently part of the culture collection of the Laboratório de Biotecnologia e Biodiversidade para o Meio Ambiente (LBBMA–UFV).

The bacterial isolates were reactivated on Tryptic Soy Agar (TSA) plates and incubated at 35 °C for 24 h. Individual colonies were then selected and transferred into 10 mL of fresh Mueller–Hinton Broth, followed by overnight incubation at 35 °C with shaking at 150 rpm. As a negative control, sterile Mueller–Hinton broth was incubated under the same conditions in parallel.

The minimum inhibitory concentration (MIC) was determined using the broth microdilution method, according to Balouiri et al. (2016), with minor modifications. Briefly, two-fold serial dilutions of the biosurfactant solutions were prepared in Mueller–Hinton broth in 96-well microtiter plates, resulting in a concentration range from 1,000 to 15.6 μg mL^{-1} (Figure 1). Each well was inoculated with a bacterial suspension adjusted to an optical density at 600 nm (OD_{600}) of 0.05. The final row of the plate contained the growth control (inoculated broth without biosurfactant) and the sterility control (non-inoculated broth).

The plates were incubated at 35 °C for 24 h. After incubation, 30 μL of resazurin solution (150 mg L^{-1}) was added to each well to assess cellular metabolic activity. A color change from blue to pink indicated bacterial growth, whereas wells that remained blue were considered to show inhibition of metabolic activity. The MIC was defined as

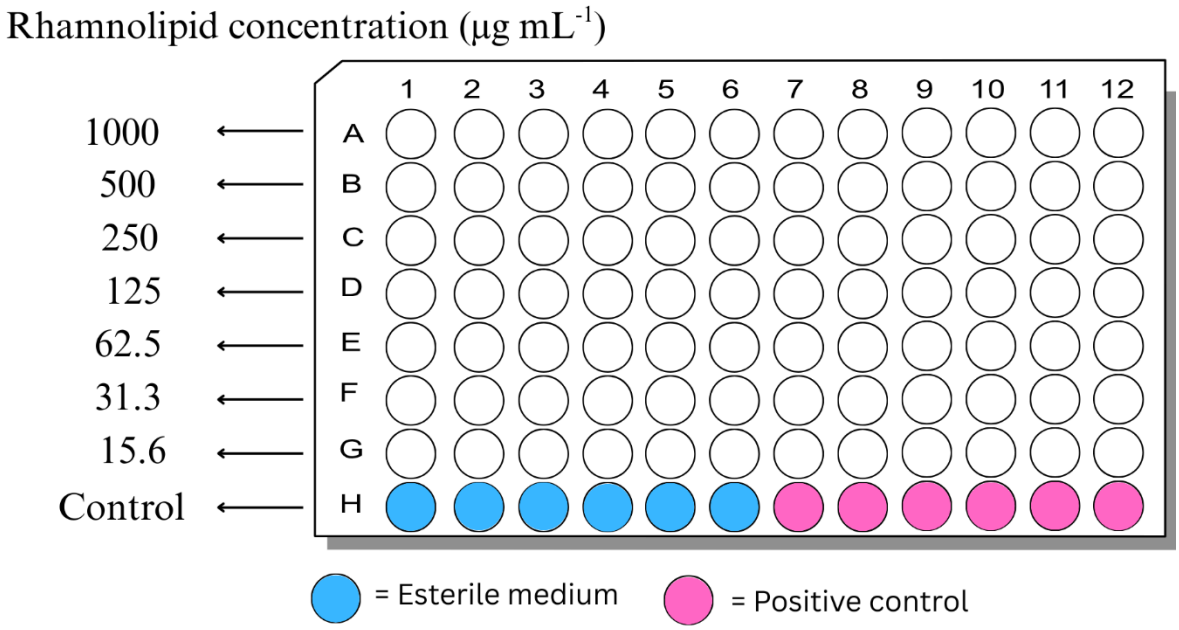
the lowest biosurfactant concentration that prevented the color change. All assays were performed in triplicate.

For data processing, optical density (OD_{600}) readings were normalized using a modified version of the methodology described by Campbell (2011) (Equation 2).

$$(2)\quad Normalized\ OD\ = \frac{OD_{sample} - \underline{OD_{neg}}}{\underline{OD_{pos}} - \underline{OD_{neg}}}$$

Where $\underline{OD_{pos}}$ and $\underline{OD_{neg}}$ represent the mean OD of the positive and negative controls, respectively. Once normalized using the equation 2, the normalized values for each strain were averaged and, posteriorly, the results of each strain were multiplied by 100 to express the results as percentage of survival (Campbell, 2011). Once obtained the percentage of survival across the different concentrations, the results were plotted as concentration-response curves on a logarithmic scale to visualize the inhibition profile for each tested strain (*E. coli* and *S. aureus*).

Figure 1. Microdilution plate layout for determining the MIC of biosurfactants against *E. coli* and *S. aureus*.



The assay was performed in 96-well microtiter plates that contains serial dilutions of biosurfactants (2000 - 15.6 µg mL^{-1}). Wells H1–H4 contained sterile medium as negative control (blue wells), and wells

H5–H8 were used as the positive control (pink wells) containing bacteria inoculum in absence of rhamnolipids. Source: authors

2.2.9 Assessment of synergism between biosurfactants and antibiotic:

2.2.9.1 Determination of MIC value in Chlomphenicol

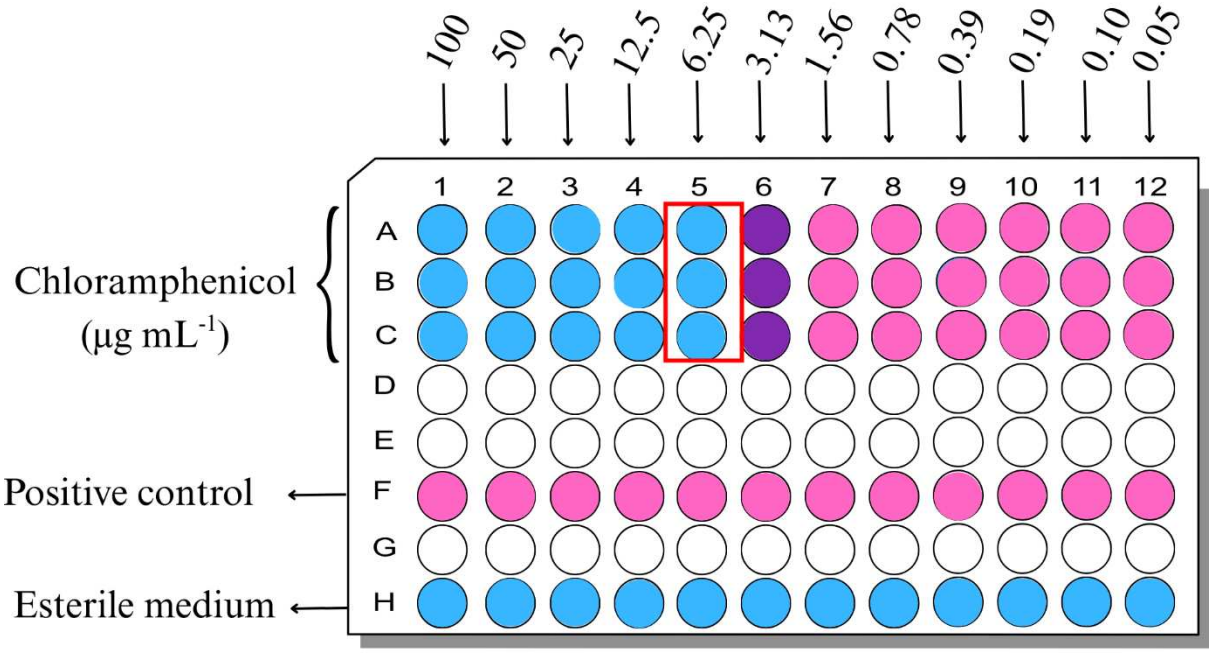
Before performing the synergy assays, it was necessary to determine the minimum inhibitory concentration (MIC) of chloramphenicol (CLO) when used alone. Therefore, the initial step consisted of evaluating the antibacterial efficacy of the antibiotic against both *Escherichia coli* and *Staphylococcus aureus*. Antibacterial activity was assessed using the broth microdilution method in 96-well microplates.

Serial two-fold dilutions of CLO were prepared to cover a wide concentration range, starting at 100 μg mL^{-1} and decreasing to a final concentration of 0.05 μg mL^{-1} (Figure 2) for each bacterial strain. Bacterial suspensions were adjusted to an optical density at 600 nm (OD_{600}) of 0.05 prior to inoculation. Each column of the microplate was used as a technical replicate, applying the same concentration range to ensure reproducibility of MIC determination.

Row F of the 96-well plate was used as a positive control (bacterial growth in sterile Mueller–Hinton broth), while row H served as the negative control (sterile medium only). Plates were incubated statically at 35 °C for 24 h. Bacterial growth was initially assessed by measuring OD_{600} and comparing the values with those obtained at the start of incubation.

To confirm the visually determined MIC, 20 μL of resazurin solution was added to each well, followed by incubation for 2 h. Colorimetric changes were then evaluated, where reduction of resazurin from blue to pink indicated active bacterial metabolism (Sarker, Nahar and Kumarasamy, 2007; Elshikh et al., 2016). The same procedure was applied to both *E. coli* and *S. aureus* strains.

Figure 2. Microdilution plate disposition for determining the MIC of chloramphenicol against *E. coli* and *S. aureus*.



Each line corresponds to a biosurfactant concentration (1000 to 15.6 µg mL^{-1}). Blue wells represent the sterile medium (negative control), and pink wells represent the growth control. The red rectangle indicates the MIC value determined for the biosurfactant treatment. Source: authors

2.2.9.2 Minimal inhibitory concentration (MIC) of chloramphenicol combined with rhamnolipids

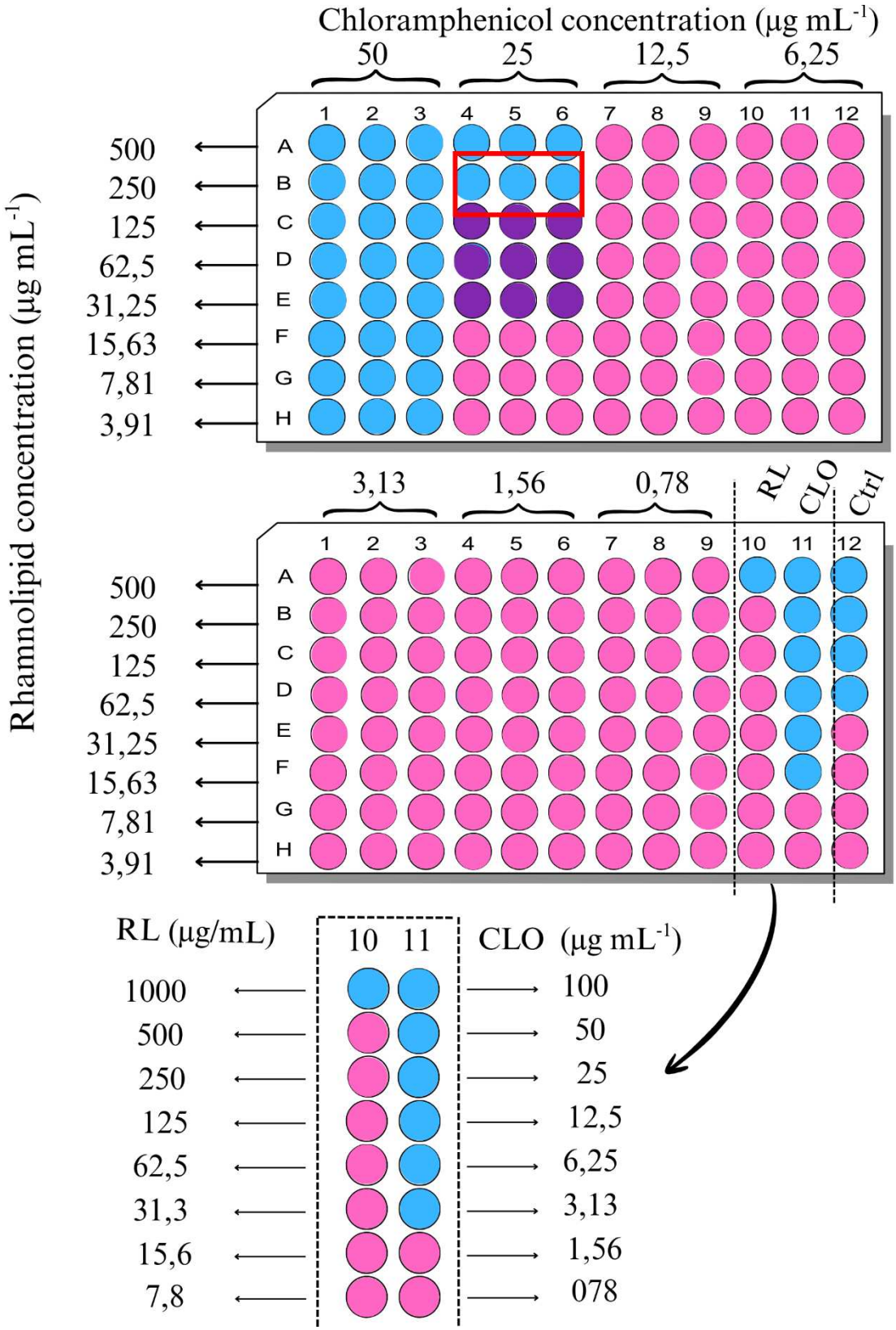
After establishing the minimum inhibitory concentration (MIC) of chloramphenicol (CLO) for both *Escherichia coli* and *Staphylococcus aureus*, synergy assays were performed to evaluate the combined antimicrobial effect of chloramphenicol and rhamnolipids using the checkerboard methodology, as described by White et al. (1996). For this assay, one biological replicate of each rhamnolipid extract was randomly selected.

Based on the previously determined MIC values of chloramphenicol, which ranged between 3 and 7 µg mL^{-1} for *E. coli* and *S. aureus*, respectively, a total of six 96-well microplates were prepared. Each plate contained Mueller–Hinton broth supplemented with one rhamnolipid extract: two plates for RL-58, two for RL-79, and two for RL-92. This experimental design was applied independently for *E. coli* and *S. aureus*, as illustrated in Figure 3.

Initial concentrations were set at 50 μg mL^{-1} for chloramphenicol and 500 μg mL^{-1} for the biosurfactants. Chloramphenicol was serially diluted horizontally across the columns of the microplates, from left to right, reaching minimum concentrations of 0.78 μg mL^{-1} for *E. coli* and 1.25 μg mL^{-1} for *S. aureus*. In contrast, the biosurfactants were serially diluted vertically across the rows, with the lowest concentration (31.2 μg mL^{-1}) located in row H.

The final three columns of each microplate were assigned specific control conditions to evaluate bacterial growth in the presence of individual agents or in their absence. Column 10 was used to assess bacterial growth in the presence of biosurfactant alone, starting at a concentration of 1,000 μg mL^{-1} and serially diluted to 7.81 μg mL^{-1}. Column 11 was designated to evaluate bacterial growth in the presence of chloramphenicol alone, beginning at 12.5 μg mL^{-1}. Column 12 was divided into two sections: one serving as a positive control (bacterial growth in Mueller–Hinton broth without chloramphenicol or biosurfactants) and the other serving as a negative control, containing sterile Mueller–Hinton broth only.

Figure 3. Experimental design of the microdilution assay used to assess the antimicrobial interaction between biosurfactants and chloramphenicol.



Serial dilutions of biosurfactants (1000 - 3.91 µg mL^{-1}) were prepared along the vertical axis, while chloramphenicol concentrations (50 - 0.78 µg mL^{-1}) were distributed along the horizontal axis. The intersection of both gradients allows the evaluation of the combined effect on bacterial growth. Blue wells represent absence of growth and pink wells correspond to growth. In the case of the ten rows from

the second microplate, were assessed the bacterial growth in presence of only biosurfactants in serial dilution (1000 - 7.8 μg mL^{-1}) and the eleven row assessed the behavior of the bacteria in the presence of only chloramphenicol (100 - 0.78 μg mL^{-1}). Finally, the twelve row of the second microplate was utilized to evaluate the sterile medium (wells 1 to 4) and the positive control (wells 5 to 8). The red rectangle indicates the MIC value determined for the biosurfactants treatment. Source: authors.

Finally, the combination effect was measured by using the equation of the fractional inhibitory concentration (3) (Holtappels et al., 2018).

$$(3)\ FIC = \left(\frac{MIC_{A\ combined}}{MIC_{A\ alone}}\right) + \left(\frac{MIC_{B\ combined}}{MIC_{B\ alone}}\right)$$

2.2.10 Suppression of fungal growth

To evaluate the antifungal potential of the biosurfactants, the present study used the plant pathogenic fungus *Corynespora cassiicola* as the target organism. The fungal isolate was provided by the Phytopathology Department of the Universidade Federal de Viçosa (UFV), Minas Gerais, Brazil. For maintenance and propagation, the fungus was cultured on Potato Dextrose Agar (PDA) plates and incubated for 13 days at 30 °C under a 12 h light/12 h dark photoperiod.

Antifungal activity was assessed using an initial biosurfactant stock solution prepared at a concentration of 4,000 μg mL^{-1} in distilled water. Serial dilutions were prepared directly in 96-well microtiter plates (Figure 4), resulting in final biosurfactant concentrations ranging from 2,000 to 31.25 μg mL^{-1}. Intermediate concentrations of 1,000, 500, 250, 125, and 62.5 μg mL^{-1} were also included.

Fungal spores were quantified using a Neubauer counting chamber and adjusted to a final concentration of 1×10^5 spores mL^{-1} in Czapek medium. This spore suspension was added to all wells except those designated as blanks. The final row of each 96-well plate was reserved for controls, including a growth control (Czapek medium inoculated with *C. cassiicola* but without biosurfactants) and a blank control (sterile Czapek medium only).

Initial optical density (OD) readings were taken at 620 nm, after which the plates were incubated at 30 °C for 8 days. Following incubation, OD_{620} measurements were

recorded again. Subsequently, 30 µL of resazurin solution was added to each well to assess metabolic activity, and the percentage of fungal growth inhibition was calculated according to Equation (3), as described by Sen et al. (2020) and Onlamool, Saimmai and Maneerat (2022).

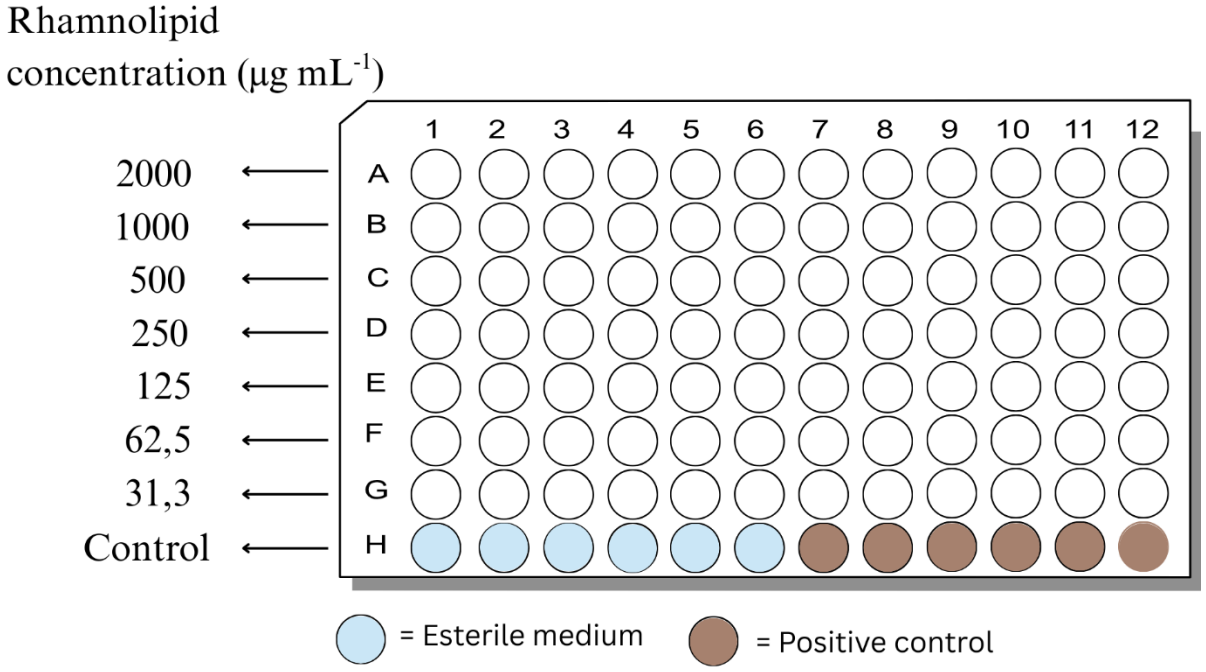
$$(4)\ Fungal\ Growth\ (\%) = \frac{OD_{treated}}{OD_{control}} x\ 100$$

Where:

$OD_{treated}$ is the optical density (or absorbance) of the treated sample.

$OD_{control}$ is the optical density (or absorbance) of the positive control (growth without treatment).

Figure 4. Schematic representation of the microdilution method used to determine the MIC of rhamnolipids against *Corynespora cassiicola.*



Blue wells represent the sterile medium (negative control), and brown wells correspond to the positive control containing fungal inoculum. To assess the reaction of *Corynespora cassiicola* in presence of biosurfactants, serial dilution of the biosurfactants were made starting in 2000 µg mL^{-1} and finishing in 31.3 µg mL^{-1}. Source: authors

2.3 Statistical analysis

All experiments, except those involving strain LBBMA 58, were performed in biological and technical triplicates, and results are expressed as mean ± standard deviation (SD). All statistical analyses were conducted using R software.

For the oil spreading assay, statistical analyses were performed to determine whether significant differences existed among the halos produced by the crude biosurfactant extracts of strains LBBMA 58, LBBMA 79, and LBBMA 92. Data normality was first evaluated using the Shapiro–Wilk test, while homogeneity of variances was assessed using Levene's test. As the data from two of the strains did not follow a normal distribution, a non-parametric Kruskal–Wallis test was applied. Once significant differences among strains were detected, Dunn's post hoc test with Bonferroni correction was used for pairwise comparisons to identify statistically significant differences between strains.

For the emulsification index (E_{24}), statistical analyses were conducted to evaluate whether significant differences existed among the emulsification performances of the three biosurfactant extracts across the tested substrates (diesel, soybean oil, and petroleum). Data distribution was first assessed using the Shapiro–Wilk test, which indicated that the emulsification values did not follow a normal distribution. Homogeneity of variances was subsequently evaluated using Levene's test and no significant differences were detected among strain–substrate combinations, indicating that the assumption of variance homogeneity was met.

Given the violation of the normality assumption, a non-parametric factorial approach was employed. Therefore, the Aligned Rank Transform (ART) method was applied to evaluate the main effects of strain and substrate, as well as their interaction, on emulsifying activity.

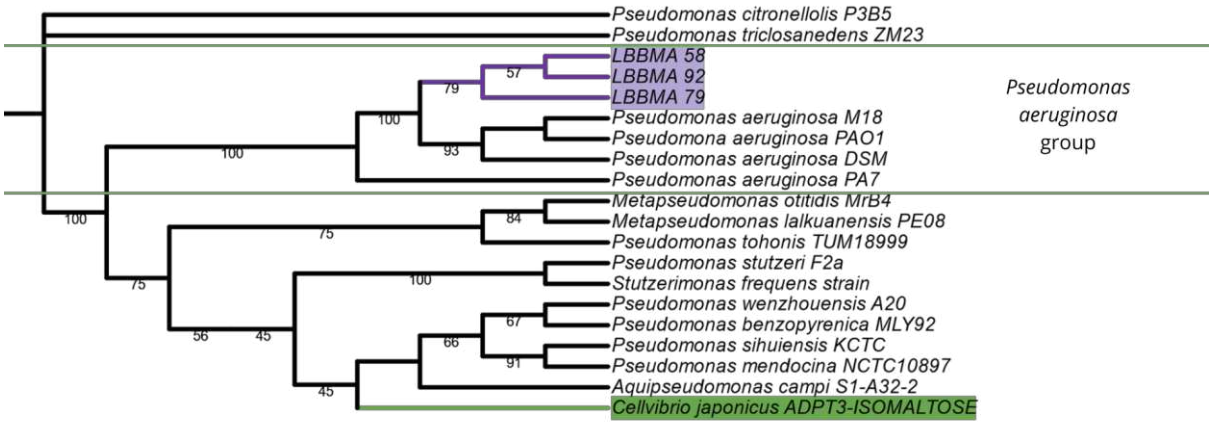
2.4 Results and discussion

2.4.1 Identification of the Pseudomonas isolates through multilocus sequence analysis (MLSA)

Analysis of the 16S rRNA, rpoD, and gyrB gene sequences revealed that strains LBBMA 58, LBBMA 79, and LBBMA 92 consistently clustered within the *Pseudomonas aeruginosa* clade, grouping with reference strains such as *P. aeruginosa* DSM, *P. aeruginosa* PAO1, and *P. aeruginosa* M18. This phylogenetic placement supports the taxonomic identification of all three isolates as *P. aeruginosa*. The robustness of this classification is further reinforced by the high bootstrap support values observed for the major nodes (>70%).

Although the three strains clustered closely within the *P. aeruginosa* group, minor sequence variations were detected among them, indicating the presence of subtle genetic differences. These variations may reflect differences in accessory or regulatory genes and could potentially influence strain-specific traits, including the synthesis of biosurfactants and other secondary metabolites.

Figure 5. Phylogenetic tree of rhamnolipid-producing *Pseudomonas* strains (LBBMA 58, LBBMA 79 and LBBMA 92) based on the *16S* rRNA, *gyrB*, and *rpoD* concatenated sequences.



The phylogenetic tree was constructed taking into account the *16S* rRNA, *rpoD* and *gyrB* concatenated sequences, of the strains LBBMA 58, 79, and 92. *Cellvibrio japonicus* ADP3 was used as the outgroup. Source: authors.

Finally, although concatenated multilocus analysis improves taxonomic resolution compared to single-gene approaches, it still provides only a partial view of the genomic diversity of these strains and does not fully capture their complete genomic context. In addition, multilocus phylogenetic reconstruction based on concatenated genes cannot adequately account for genomic events such as horizontal gene transfer or chromosomal rearrangements, which play a key role in shaping the ecology, adaptability, and evolution of bacterial genera, including *Pseudomonas*.

Future studies employing whole-genome sequencing would allow a more comprehensive characterization of the LBBMA strains and provide greater resolution for confirming their taxonomic identity. Furthermore, complementary transcriptomic and metabolomic approaches would help to link genetic variation with phenotypic expression, offering deeper insights into regulatory mechanisms and metabolic potential. Together, these strategies would strengthen phylogenetic and taxonomic precision and enhance our understanding of the functional capabilities of the LBBMA strains.

2.4.2 Biosurfactant production

Quantification of the crude biosurfactant extracts revealed pronounced variability in production capacity among the *Pseudomonas aeruginosa* LBBMA strains. Strain LBBMA 92 exhibited the highest biosurfactant yield, followed by strain LBBMA 79 (214.96 ± 109.15 mg L^{-1}). However, LBBMA 92 also showed the greatest variability among replicates (711.48 ± 260.43 mg L^{-1}). In contrast, strain LBBMA 58 presented the lowest biosurfactant yield and the smallest standard deviation (130.06 ± 3.85 mg L^{-1}), suggesting a more consistent metabolic production profile under the tested conditions. It should be noted, however, that for strain LBBMA 58 only two biological replicates were obtained, whereas three replicates were available for strains LBBMA 79 and LBBMA 92. The reduced number of replicates for LBBMA 58 was due to sample loss caused by handling errors during processing. For this reason, throughout the present work, data corresponding to strain LBBMA 58 are presented exclusively as replicate 1 (58-1) and replicate 2 (58-2). The higher variability observed in strains LBBMA 79 and LBBMA 92 may therefore reflect differences in metabolic regulation or growth dynamics, even when cultivated under identical experimental conditions.

For strain LBBMA 92, the production values obtained are consistent with the maximum yields reported during optimization studies of *P. aeruginosa* by Araujo et al. (2020), which ranged from 300 to 840 mg L^{-1}. In contrast, strains LBBMA 58 and LBBMA 79 exhibited substantially lower production levels, suggesting that these strains are less efficient rhamnolipid producers than LBBMA 92 under the conditions evaluated in this study.

It is important to note that high rhamnolipid yields in *P. aeruginosa* are commonly achieved when hydrophobic carbon sources, particularly vegetable oils, are used. Low-cost substrates such as vegetable oils or agro-industrial residues are frequently reported as highly efficient for rhamnolipid production due to their ability to activate the β-oxidation pathway. However, the objective of the present study was not to optimize production yield or cost efficiency. Instead, glucose was intentionally selected as the carbon source because it provides a chemically defined, controlled, and highly reproducible substrate, which is essential for comparative and mechanistic analyses.

The use of oil-based substrates can introduce variability related to substrate composition, emulsification behavior, and extraction artifacts, which may interfere with downstream physicochemical and biological characterization of the produced biosurfactants. In contrast, glucose-based media favor the production of cleaner rhamnolipid extracts, minimizing matrix effects and ensuring that observed differences among strains primarily reflect biological variability rather than substrate complexity.

Therefore, glucose was chosen to ensure standardized cultivation conditions and to support the primary objective of this work: the characterization and comparison of rhamnolipids produced by different *Pseudomonas* strains under well-defined experimental conditions, rather than the maximization of production efficiency.

2.4.3 Characterization and quantification of biosurfactants

The analysis of the biosurfactants by tandem mass spectrometry (MS/MS) revealed that all the *P. aeruginosa* LBBMA strains produced rhamnolipids (Table 2). The results were compared with the literature to identify the rhamnolipid congeners present in each sample, considering how many ions are weighted in relation to their electric charge (m/z ratio) (Table 2). For this purpose, the articles of Zhu et al. (2022) and Abdel-Mawgoud, Lépine and Déziel (2010) were used as references. Additionally, based on the results, the generated area was established by summing the different transition ions for each molecule.

Table 2. Rhamnolipid congeners identified by tandem mass spectrometry (MS/MS) and their respective electric charge (m/z).

Compound	**m/z**
Rha-C10-C10	504
Rha-C10-C12	530
Rha-Rha-C10-C10	650

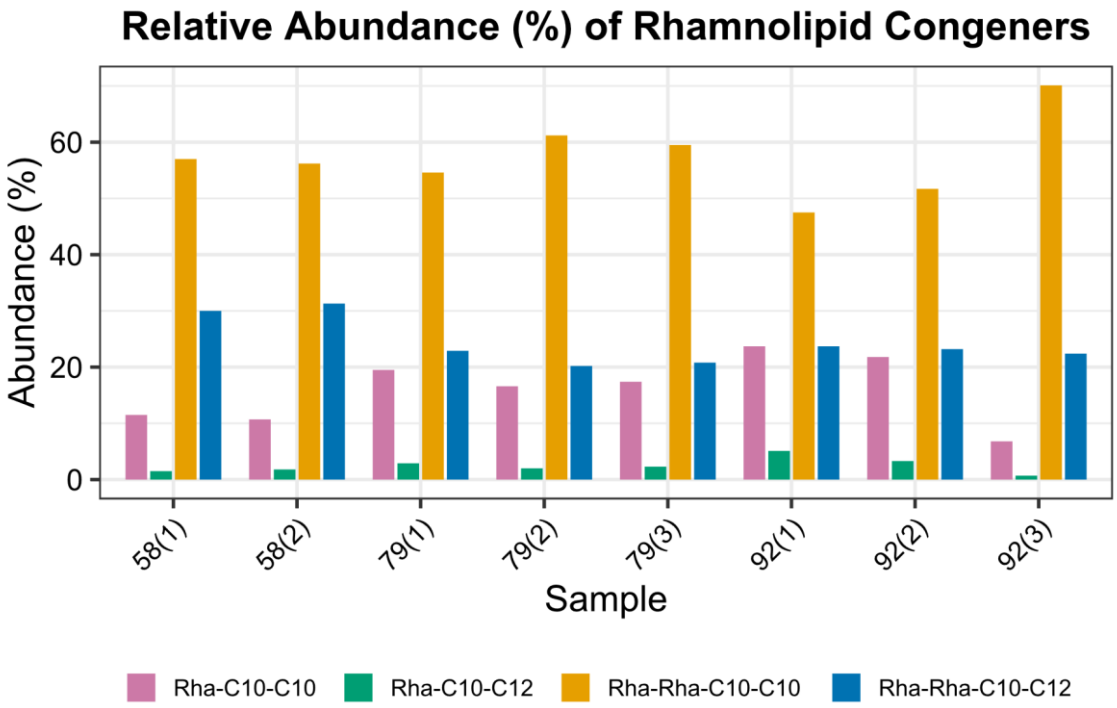
Rha-Rha-C10-C12	678

The relative abundance of rhamnolipid congeners is summarized in Figure 6. Across all strains, the predominant rhamnolipid congener was Rha–Rha–C10–C10 (Figures 6 and 7), consistently representing the largest fraction (>60%) in each profile. Similarly, analysis of the commercial rhamnolipid standard revealed a clear predominance of di-rhamnolipids. These findings are consistent with previous reports describing the preferential production of di-rhamnolipids—particularly Rha–Rha–C10–C10—by species of the genus *Pseudomonas* (Christova et al., 2011; Dos Santos, 2020; El-Housseiny et al., 2020).

In contrast, the congeners Rha–C10–C10 and Rha–Rha–C10–C12 were detected at relatively constant intermediate levels across samples, whereas Rha–C10–C12 consistently appeared as the least abundant congener. This pattern suggests that the biosynthetic machinery favors di-rhamnolipids containing two C10 fatty acid chains, while congeners with longer acyl chains, such as Rha–C10–C12, are less favored during biosynthesis.

The overall similarity in congener distribution among the three laboratory strains indicates a conserved biosynthetic balance, characterized by preferential synthesis of Rha–Rha–C10–C10 and reduced production of alternative congeners.

Figure 6. Relative abundance of Rhamnolipid congeners of eight samples and a commercial rhamnolipid.



The figure shows the relative proportions of the four rhamnolipid congeners found in the samples — Rha-C10-C10, Rha-C10-C12, Rha-Rha-C10-C10, and Rha-Rha-C10-C12—synthezised by the strains LBBMA 58, LBBMA 79, and LBBMA 92. Data are expressed as mean relative abundance (%). Source: authors

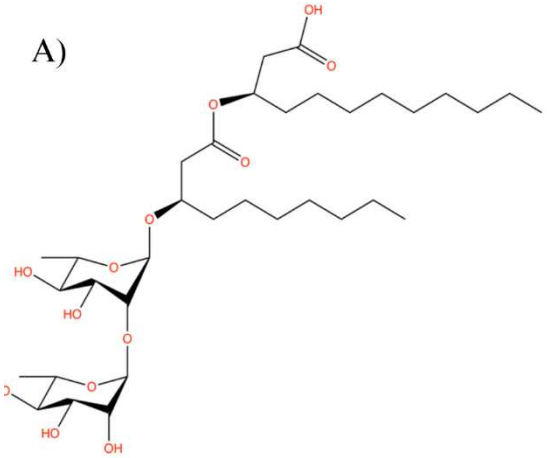
Naturally produced rhamnolipids consist of mixtures of different rhamnolipid congeners present in varying proportions (Abdel-Mawgoud, Lépine and Déziel, 2010; El-Housseiny et al., 2020; Cerqueira dos Santos et al., 2024). These complex mixtures allow interactions among congeners, conferring distinctive physicochemical properties to the overall biosurfactant system (Abdel-Mawgoud, Lépine and Déziel, 2010). As shown in Figure 6, each strain exhibited slight differences in the relative abundance of individual congeners. Notably, the third replicate of strain LBBMA 92 (RL-92(3)) displayed a congener distribution that differed slightly from those observed in replicates RL-92(1) and RL-92(2).

Such variations are not unexpected and reflect the inherent biological variability associated with living organisms and, consequently, with their metabolic outputs. Current evidence indicates that the relative abundance of rhamnolipid congeners can be influenced by multiple factors, including intrinsic metabolic fluctuations, quorum

sensing dynamics, and environmental heterogeneity, among others (Chong and Li, 2017; Kłosowska-Chomiczewska et al., 2024).

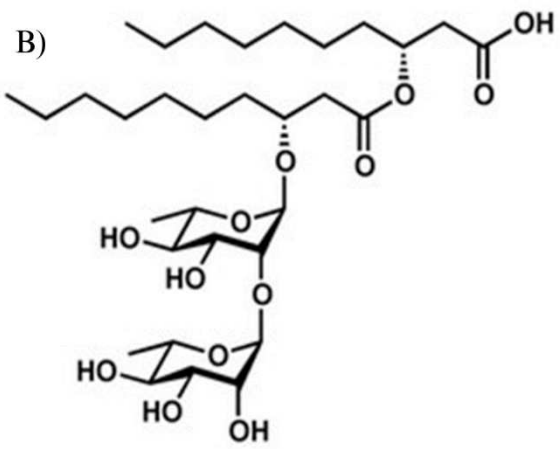
Figure 7. Chemical structures of rhamnolipid congeners identified by tandem mass spectrometry (MS/MS).

A)



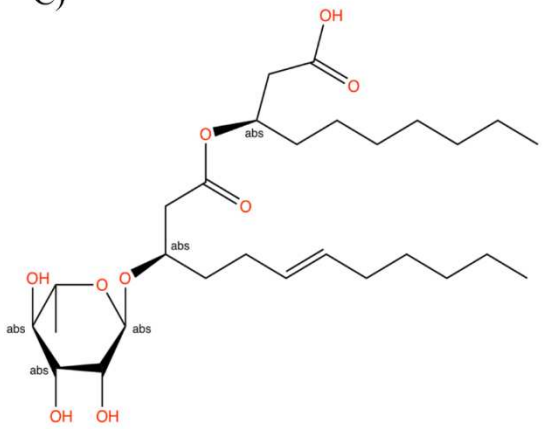
RHA-RHA-C10-C12
Average molar mass: 678.86
Transition ions: 8

B)



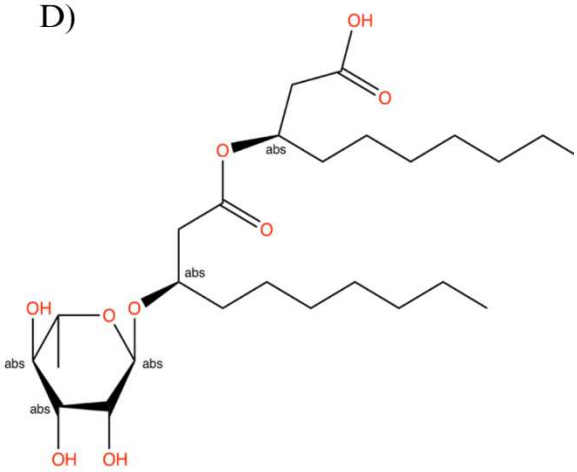
RHA-RHA-C10-C10
Average molar mass: 650.8
Transition ions: 6

C)



RHA-C10-C12
Average molar mass: 530.69
Transition ions: 4

D)



RHA-C10-C10
Average molar mass: 504.65
Transition ions: 3

(A) Rha-Rha-C10-C12; (B) Rha-Rha-C10-C10; (C) Rha-C10-C12; (D) Rha-C10-C10. Molecular weights and transition ions correspond to values obtained experimentally. Based on the mass-to-charge (m/z) ratios obtained from the MS/MS analysis, each congener was identified according to the reference data reported by Zhu et al. (2022). Source: Zhu et al. (2022).

As shown in Figure 6, variability was observed not only among different strains but also among replicates of the same strain. This variability can be attributed to both technical inconsistencies inherent to laboratory procedures and intrinsic biological heterogeneity. Even under controlled experimental conditions, it is not possible to maintain all environmental parameters completely identical across replicates. Subtle fluctuations in temperature, pH, oxygen availability, or agitation may significantly influence microbial performance and metabolite production. In addition, experimental procedures are inherently subject to minor human-related deviations during sample handling and execution, which can contribute to variability among replicates. Therefore, a certain degree of technical variation is unavoidable in biological experiments.

Beyond technical factors, the observed variability can also be explained by biological heterogeneity governed by genetic and regulatory mechanisms associated with rhamnolipid biosynthesis. Rhamnolipid production is mediated by three key genes: *rhlA*, *rhlB*, and *rhlC*. The gene *rhlA* encodes the enzyme 3-(hydroxyalkanoyloxy)alkanoate synthase (HAA synthase), which catalyzes the formation of fatty acid dimers that serve as precursors for mono-rhamnolipid synthesis by rhamnosyltransferase I, encoded by *rhlB*. Di-rhamnolipids are subsequently produced by rhamnosyltransferase II, encoded by *rhlC*. Importantly, *rhlA* and *rhlB* are co-expressed within the same operon (*rhlAB*), whereas *rhlC* is located in a separate operon (Schmidberger et al., 2013). This genetic organization can influence both the quantity and the relative proportions of rhamnolipid congeners produced.

However, rhamnolipid synthesis is not determined solely by the expression of *rhlA*, *rhlB*, and *rhlC*. It is also strongly influenced by precursor availability, including fatty acids and activated sugar units (dTDP-L-rhamnose). In addition, rhamnolipid biosynthesis is tightly regulated by growth-dependent quorum sensing (QS) systems, nutritional conditions, and virulence-associated regulatory networks (Schmidberger et al., 2013; Bahia et al., 2018). Quorum sensing itself is subject to further modulation, for example through *rsaL*, which acts as a negative regulator of QS-controlled genes (Rampioni et al., 2006). Together, these layers of regulation contribute to the biological variability observed in congener profiles and biosurfactant yields.

Oil spreading technique

Table 3. Oil spreading activity of rhamnolipid extracts produced by *Pseudomonas* strains LBBMA 58, LBBMA 79, and LBBMA 92.

Sample	Oil spreading (cm)
RL-92	7.39 ± 2.27
RL-79	5.08 ± 1.58
RL-58	2.1 ± 0.2
Blank sample (medium without cell growth)	0.00

Biosurfactants such as rhamnolipids are capable of markedly reducing surface and interfacial tension between immiscible phases, such as aqueous and hydrophobic systems (Chen, Juang and Wei, 2015). When a droplet of a biosurfactant solution is applied to an oil film, surfactant molecules rapidly migrate to the water–oil interface, disrupting cohesive forces and lowering interfacial tension. This process results in the formation of a characteristic clear halo, which is the basis of the oil spreading technique. This physicochemical property underlies the broad applicability of rhamnolipids across several fields, particularly environmental and pharmaceutical applications.

In environmental contexts, the capacity of rhamnolipids to mobilize hydrophobic compounds is especially valuable. This property can be exploited in enhanced oil recovery processes, where biosurfactants facilitate the mobilization of oil trapped within porous rock matrices. Similarly, in oil spill remediation, rhamnolipids can enhance oil dispersion, thereby reducing damage to both aquatic and terrestrial ecosystems. In addition, by increasing the solubility and bioavailability of hydrophobic pollutants, biosurfactants promote microbial access to hydrocarbons and can significantly accelerate biodegradation processes (Banat et al., 2010).

In the pharmaceutical field, the ability of rhamnolipids to solubilize or encapsulate poorly water-soluble compounds makes them promising candidates for drug delivery systems. By improving the transport and bioavailability of hydrophobic therapeutic agents, rhamnolipids may enhance drug efficacy and reduce required dosages, contributing to more efficient and targeted therapeutic strategies (Bjerk et al., 2021)

2.4.5 Determination of Critical Micelle Concentration

One of the most important physicochemical parameters used to characterize rhamnolipids is the critical micelle concentration (CMC) (Perinelli et al., 2020). Conceptually, the CMC represents the surfactant concentration at which micelles begin to form in solution, marking the transition between monomer dominated and micelle dominated states (Vittal, Gomathi and Kim, 2006; Abooali and Soleimani, 2023). From an experimental perspective, the CMC is defined as the minimum concentration of surfactant required to reduce the surface tension of water to its lowest attainable value, beyond which further increases in surfactant concentration no longer result in significant surface tension reduction (Ramesh and Sakthishobana, 2021).

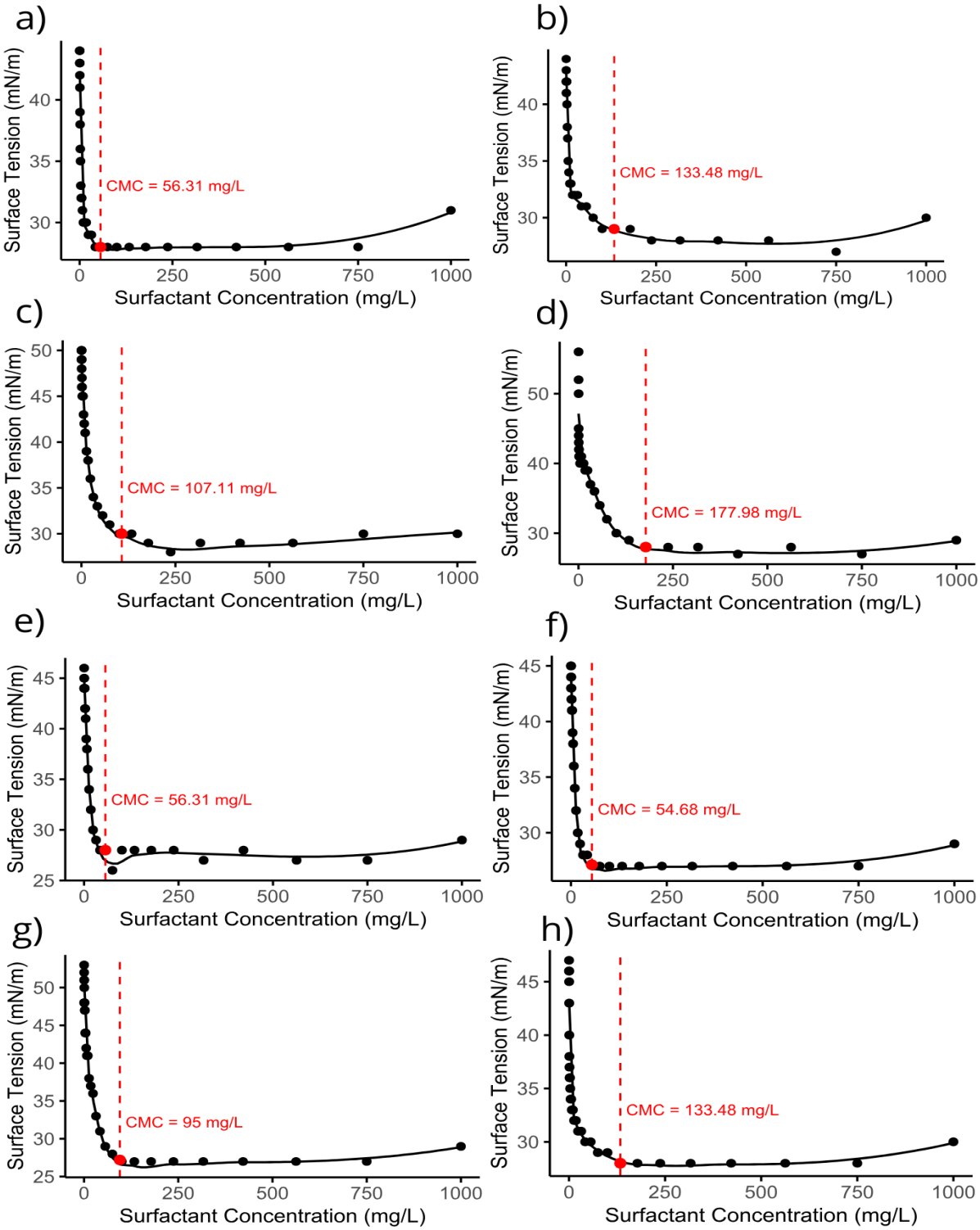
Determination of the CMC is therefore a key indicator of surfactant efficiency, as lower CMC values reflect a higher surface activity and reduced material requirements to achieve effective interfacial tension reduction. This parameter allows direct comparison between synthetic and biological surfactants and facilitates the selection of the most suitable compound for specific applications.

It is important to note that the CMC is not a fixed value but rather a dynamic parameter influenced by several environmental and molecular factors. Variables such as temperature, pH, and ionic strength can significantly affect micelle formation and stability (Özdemir, Peker and Helvaci, 2004; Tennouga et al., 2015). In addition, the molecular structure of the surfactant, particularly hydrophobic chain length, degree of saturation, and head group composition plays a critical role in determining the CMC.

As illustrated in Figure 8, the progressive addition of surfactant to water leads to a gradual decrease in surface tension until a plateau is reached. This inflection point, where surface tension no longer decreases despite increasing surfactant

concentration, is operationally defined as the CMC (Ramesh and Sakthishobana, 2021).

Figure 8. Determination of the critical micelle concentration (CMC) of rhamnolipids produced by *Pseudomonas* strains (LBBMA 58, LBBMA 79 and LBBMA 92) based on surface tension measurements



The figure shows the surface tension (mN m^{-1}) as a function of surfactant concentration (mg L^{-1}) for each rhamnolipid extract and replicate, where (a) LBBMA 58(1), (b) LBBMA 58(2), (c) LBBMA 79(1), (d) LBBMA 79(2), (e) LBBMA 79(3), (f) LBBMA 92(1), (g) LBBMA 92(2), and (h) LBBMA 92(3). The CMC value was indicated using the red dashed lines and each curve represents the mean of triplicate measurements. Source: authors

As shown in Figure 8, all eight rhamnolipid extracts effectively reduced the surface tension of water from an initial value of 72 mN m^{-1} to values ranging between 27.33 and 29.97 mN m^{-1}. According to Table 4, strain LBBMA 92 exhibited the best overall performance, particularly in replicate 1, which achieved the lowest surface tension value (27.33 mN m^{-1}) at a CMC of 54.68 mg L^{-1}.

For strain LBBMA 58, both replicates reached surface tension values below 29 mN m^{-1} (27.95 mN m^{-1} and 28.85 mN m^{-1}); however, their CMC values differed substantially (56.31 mg L^{-1} and 133.48 mg L^{-1}, respectively). In contrast, strain LBBMA 79 showed the highest surface tension values at the CMC, ranging from approximately 28 to 30 mN m^{-1}, and also displayed the greatest variability among replicates, with CMC values spanning from 56.31 to 177.98 mg L^{-1}.

The variability observed both among strains and between biological replicates likely reflects a combination of biological and technical factors. Biological variability may arise from fluctuations in metabolic activity, regulatory pathways, and, most critically, from differences in the relative abundance of rhamnolipid congeners. As shown in Figure 6, the congener profiles of the three strains are not identical; for instance, RL-79 exhibits a higher proportion of the mono-rhamnolipid Rha-C10-C10 compared to RL-58 and RL-92. This structural variation is known to significantly influence micellization behavior. Studies have demonstrated that the critical micelle concentration (CMC) is directly correlated with the molecular structure of rhamnolipids (2, 5). Di-rhamnolipids, such as Rha-Rha-C10-C10, generally exhibit lower CMCs than their mono-rhamnolipid counterparts due to their larger headgroup, which affects their packing parameter and self-assembly properties (1, 4). Furthermore, the length and saturation of the fatty acid chains also play a role; rhamnolipid mixtures with a higher proportion of unsaturated fatty acids tend to form micelles at higher concentrations, resulting in elevated CMC values (2, 7). Therefore, the subtle differences in congener distribution observed among the strains and replicates (Figure 6) likely contribute to the broad range of CMC values obtained (from 54.68 to 177.98 mg L^{-1}), as each mixture possesses distinct hydrophilic-lipophilic balance and self-aggregation tendencies. In addition to these biological factors, technical variability inherent to surface tension measurements must be considered. Factors such as instrument calibration, tensiometer sensitivity, temperature stability, cleanliness of the measurement vessel, and minor differences in sample preparation

or dilution can influence CMC determination, particularly because CMC estimation is highly sensitive to small changes in surface tension near the inflection point of the curve.

Overall, the rhamnolipids produced in this study exhibited CMC values and surface tension reductions that are comparable to, or superior to, those reported in the literature. For example, El-Housseiny et al. (2020) and Hosseini et al. (2024) reported surface tension reductions to approximately 36 mN m^{-1} and 34 mN m^{-1}, respectively, but required higher biosurfactant concentrations (around 200 mg L^{-1}). In contrast, the rhamnolipids evaluated here achieved substantially lower surface tension values at lower concentrations.

The surface tension values obtained are consistent with previous studies reporting efficient rhamnolipid activity. Li et al. (2022) and Araujo et al. (2020) observed reductions in surface tension from approximately 72 mN m^{-1} to 28.5 mN m^{-1} and from 71.94 mN m^{-1} to 29.42 mN m^{-1}, respectively. However, while the CMC values reported by Li et al. (2022) ranged between 57.2 and 65.2 mg L^{-1} and Araujo et al. (2020) reported a CMC of 49.64 mg L^{-1} with lower variability, the present study shows a broader range of CMC values among strains and replicates, highlighting strain-dependent performance.

These results demonstrate the high efficiency of the crude rhamnolipid extracts produced by strains LBBMA 58, LBBMA 79, and LBBMA 92. Biosurfactants are generally considered effective when they are capable of reducing the surface tension of water below 40 mN m^{-1} (Hosseini et al., 2024), a threshold surpassed by all extracts evaluated in this study. Furthermore, the rhamnolipids produced here outperformed two widely used synthetic surfactants, sodium dodecyl sulfate (SDS) and cetylpyridinium bromide (CPB), in terms of efficiency. According to Elarbi et al. (2020), SDS reduced surface tension to approximately 39 mN m^{-1} only at concentrations above 2,000 mg L^{-1}, while CPB required concentrations exceeding 200 mg L^{-1} to achieve surface tension values between 37.9 and 39.9 mN m^{-1}. In contrast, the rhamnolipids evaluated in the present study achieved substantially lower surface tension values at markedly lower concentrations.

Taken together, these findings confirm the potent surface-active properties of microbial rhamnolipids and highlight their superior efficiency compared with conventional

synthetic surfactants, positioning them as highly effective and promising alternatives for industrial, environmental, and biomedical applications.

2.4.6 Determination of emulsification index (E24)

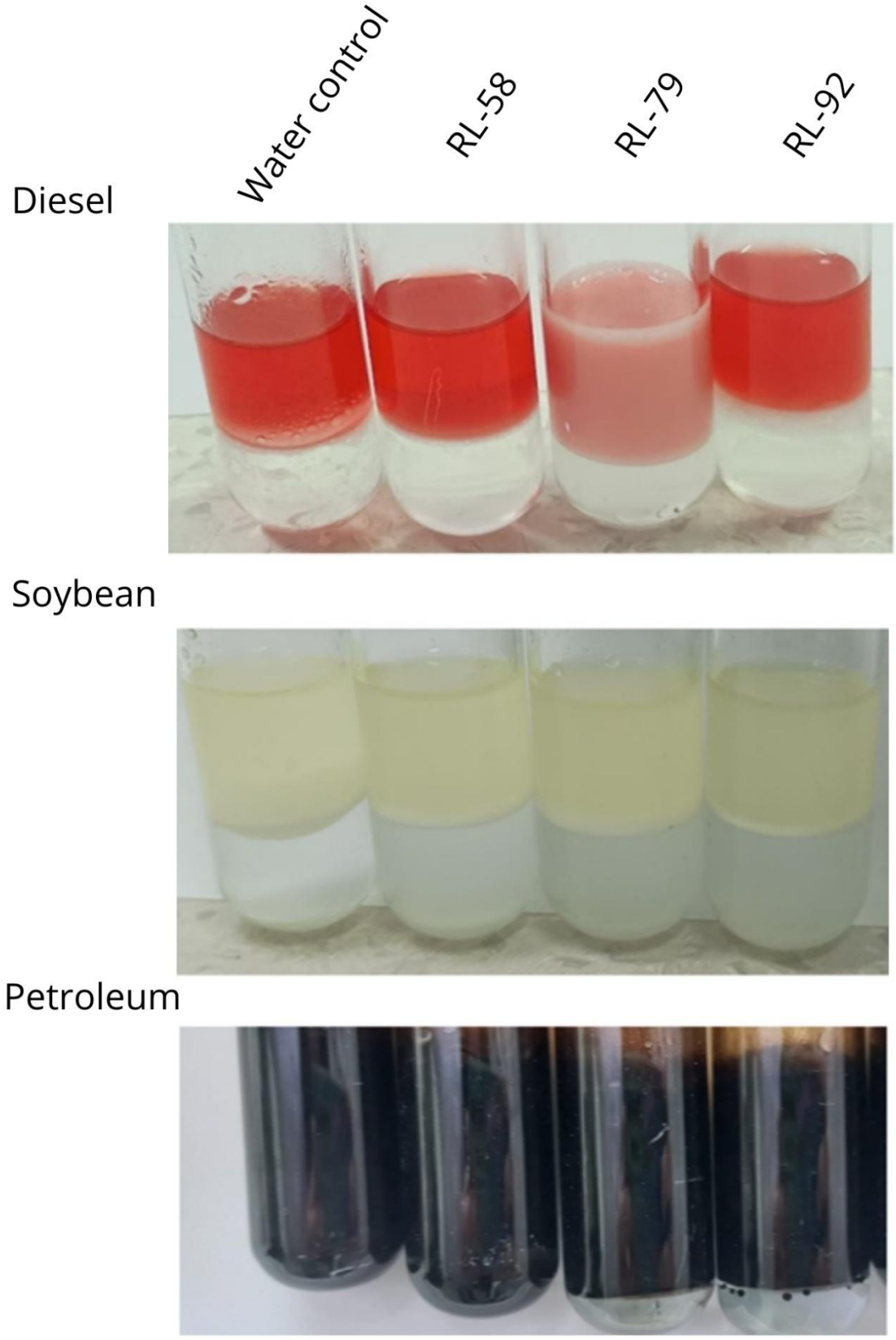
The emulsification index after 24 h (E24) is a widely used semi-quantitative parameter for assessing the ability of biosurfactants to form and stabilize oil–water emulsions over time. In contrast to surface tension measurements, which reflect interfacial activity, the E24 index provides insight into the emulsifying capacity and long-term stability of the emulsion. For this reason, E24 is particularly relevant for evaluating the applicability of biosurfactants in processes that require sustained emulsion formation, such as bioremediation of hydrophobic pollutants, enhanced oil recovery, and food, cosmetic, and pharmaceutical formulations involving immiscible phases.

The emulsification results revealed a clear substrate-dependent behavior for each crude rhamnolipid extract (RL). When petroleum was used as the hydrophobic phase (Figure 9), the rhamnolipids produced by strain LBBMA 58 (RL-58) achieved complete emulsification (100%), a value comparable to that observed for the blank control (distilled water). In contrast, RL-79 and RL-92, produced by strains LBBMA 79 and LBBMA 92, respectively, exhibited lower emulsification indices, reaching 74.73 ± 7.06% and 89.00 ± 9.28%, respectively.

For diesel and soybean oil (Figure 10), the emulsification indices were considerably lower for all rhamnolipid extracts. Among the strains, LBBMA 79 exhibited the highest emulsifying activity for both diesel (53.15 ± 2.82%) and soybean oil (8.67 ± 4.05%). Conversely, the lowest emulsification values for both substrates were observed for RL-58, produced by strain LBBMA 58, with values of 3.64 ± 0.82% for diesel and 2.16 ± 1.13% for soybean oil.

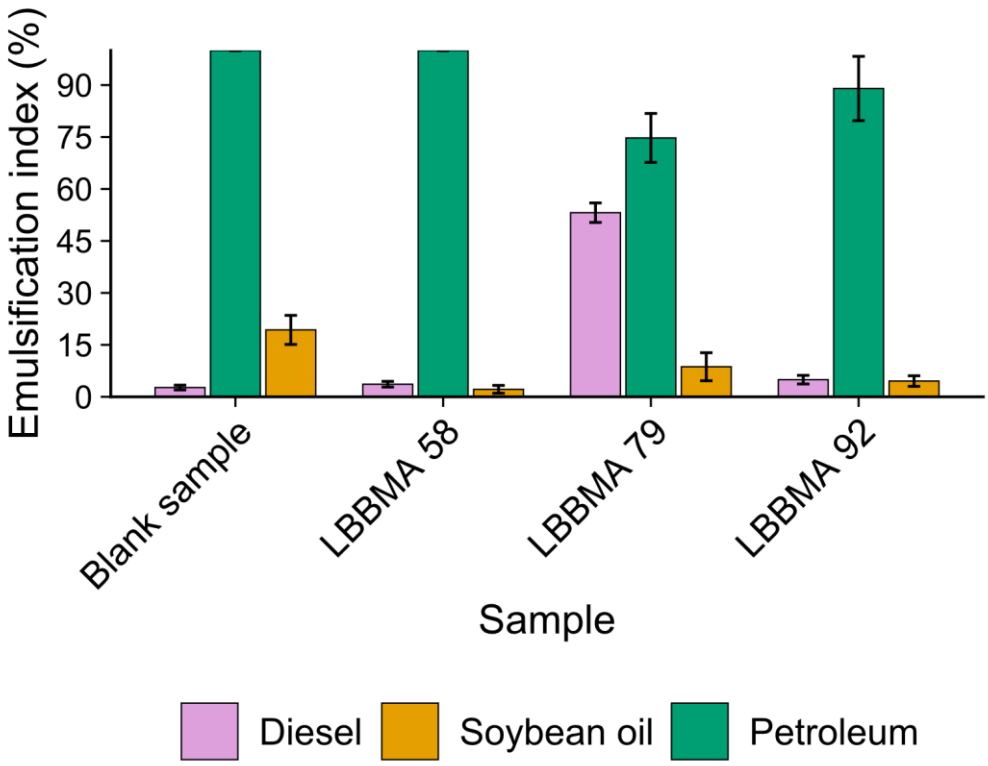
These results demonstrate that the emulsifying performance of rhamnolipids is strongly influenced by the nature of the hydrophobic substrate and varies among producing strains, highlighting the importance of substrate specific evaluation when considering potential industrial or environmental applications.

Figure 9. Emulsification index (E24) test of rhamnolipid crude extracts using diesel, soybean oil and petroleum as substrates.


Water control
RL-58
RL-79
RL-92
Diesel
Soybean
Petroleum

Visual comparison of emulsions formed after 24 h. (A) Diesel, (B) soybean oil and (C) petroleum. From left to right: distilled water (blank control), rhamnolipid crude extract from strains LBBMA 58, LBBMA 79, and LBBMA 92.

Figure 10. Evaluation of the emulsification index (E24 %) of rhamnolipid crude extracts produced by *Pseudomonas* strains against different hydrophobic substrates.



The emulsification index (E24 %) was calculated after 24 h at room temperature using diesel (purple), soybean oil (yellow), and petroleum (green) as test oils. Bars represent the mean ± SD of triplicate measurements. The blank sample (distilled water) served as the negative control. Source: authors

To determine whether significant differences existed among the rhamnolipid extracts (RLs) and the tested substrates (diesel, soybean oil, and petroleum), the statistical assumptions were first evaluated. The Shapiro–Wilk test indicated that the emulsification data did not follow a normal distribution ($W = 0.830$, $p < 0.001$). In contrast, Levene's test revealed no significant differences in variance among the

different strain–substrate combinations, confirming homogeneity of variances. Given the violation of normality, a non-parametric factorial analysis of variance was performed using the Aligned Rank Transform (ART) approach.

The ART analysis revealed statistically significant effects of rhamnolipid extract ($p < 0.001$), substrate type ($p < 0.001$), and the interaction between rhamnolipid extract and substrate ($p < 0.001$). These results clearly demonstrate that the emulsifying activity of the rhamnolipids is jointly dependent on both the producing strain and the physicochemical characteristics of the substrate. This behavior is likely explained by (i) differences in the relative abundance of rhamnolipid congeners produced by each strain and (ii) intrinsic properties of each substrate, such as viscosity, molecular composition, and hydrophobicity.

This pronounced substrate-dependent behavior highlights the potential for selective application of each rhamnolipid extract across different industrial and environmental contexts. In petroleum-related applications, emulsification can be either advantageous or undesirable depending on the process. In the context of oil spill bioremediation, emulsification is beneficial because it increases the surface area of hydrocarbons and alters substrate solubility, thereby enhancing microbial accessibility and biodegradation rates (Mulligan, 2005). This observation is consistent with previous studies reporting enhanced biodegradation of petroleum hydrocarbons following biosurfactant application (Zhang et al., 2005; Goveas et al., 2024).

Conversely, in refinery operations, the formation of stable water–oil emulsions is often undesirable, as it increases processing complexity and operational costs associated with emulsion breaking. Rhamnolipids, in particular, can form highly stable emulsions, which, although advantageous in environmental applications, represent a technical challenge in refining processes (Sousa, Pereira and Matos, 2022). In this context, the use of rhamnolipids such as RL-79 and RL-92, which exhibited lower emulsifying activity compared to RL-58, may be more appropriate.

Consistent with this substrate specificity, diesel emulsification showed marked differences among the rhamnolipid extracts. The rhamnolipid produced by strain LBBMA 79 (RL-79) achieved an emulsification index of approximately 53%, which is slightly lower than values reported by Das, Yang and Ma (2014), where Pseudomonas-

derived biosurfactants emulsified diesel in the range of 60–80%. The ability of RL-79 to emulsify diesel may be associated with the relatively lower viscosity and hydrocarbon chain composition of diesel. However, the fact that only RL-79 achieved substantial emulsification remains unclear. Minor variations in congener composition—particularly in the balance between mono- and di-rhamnolipids and fatty acid chain length—are known to strongly influence emulsifying performance. Indeed, RL-79 exhibited a higher relative abundance of mono-rhamnolipids (Rha-C10-C10) and substantial levels of the di-rhamnolipid Rha-Rha-C10-C10 (Figure 6), which together may enhance its emulsification efficiency. In contrast, RL-58 and RL-92 exhibited very low emulsification of diesel, with values of 3.64 ± 0.82% and 4.95 ± 1.24%, respectively.

For soybean oil, all rhamnolipid extracts exhibited poor emulsifying performance, with values ranging from 2.16 ± 1.13% (RL-58) to 8.67 ± 4.05% (RL-79). Interestingly, the blank control (distilled water) exhibited a higher apparent emulsification index (19.31 ± 4.20%). These results suggest that the rhamnolipids produced by strains LBBMA 58, LBBMA 79, and LBBMA 92 have limited affinity for triglyceride-rich substrates such as soybean oil, likely due to mismatches between rhamnolipid congener structure and the physicochemical properties of the oil.

2.4.7 Minimum inhibitory concentration (MIC)

2.4.7.1 Determination of MIC value in chloramphenicol

Staphylococcus aureus, a Gram-positive bacterium, and *Escherichia coli*, a Gram-negative bacterium, are globally recognized opportunistic pathogens responsible for recurrent infections in both humans and animals. In the present study, both bacterial isolates were obtained from goats diagnosed with mastitis. In the first case, mastitis was associated with *Staphylococcus aureus* infection, whereas in the second case, mastitis was caused by *Escherichia coli*. To determine whether these isolates exhibited resistance (R) or susceptibility (S) to commonly used antibiotics, their responses to a panel of antimicrobial agents were evaluated, as previously described by Silva (2025). The results of the antimicrobial susceptibility tests are presented in Tables 5 and 6.

Table 5. Antimicrobial susceptibility profile of the *Escherichia coli* isolates obtained from goat mastitis.

Antibiotic name	Halo (mm)	Parameter	Results
Cloramphenicol	25.5	R<12; S>18	S
Cefepime	22	R<14<S	S
Tetracycline	26	R<19<S	S
Gentamicin	17.5	R<17<S	S
Erythromicin	15.5	R<11; S>15	S
Enoxacin	29.5	R<16; S>23	S
Ampicilin	18.5	R<14<S	S

S = Susceptible; R = Resistant; interpretive criteria based on CLSI and BrCAST. Data information extracted from Thiago et al. (2025).

Table 6. Antimicrobial susceptibility profile of the *S. aureus* isolates obtained from goat mastitis.

Antibiotic name	Halo (mm)	Parameter	Results
Cloramphenicol	26.5	R<16; S>18	S

Penicillin	6	R<14; S>18	R
Tetracycline	25	R<19<S	S
Enoxacin	28	R<16; S>23	S
Azythromicin	17.5	R<14; S>18	I

S = Susceptible; R = Resistant; I = Intermediate susceptibility interpretive criteria based on CLSI and BrCAST, Data information extracted from Thiago et al. (2025).

Once the antimicrobial susceptibility profiles of *Escherichia coli* and *Staphylococcus aureus* were established, chloramphenicol was selected as the commercial antibiotic for subsequent experiments, based on the susceptible phenotype exhibited by both isolates. Subsequently, the minimum inhibitory concentration (MIC) of chloramphenicol was determined for each bacterium using the broth microdilution method. To facilitate the detection of bacterial growth, 20 µL of resazurin solution was added as a redox viability indicator. Resazurin is reduced by metabolically active bacterial cells, resulting in a color change from blue to pink, thereby allowing visual identification of growth inhibition.

The results revealed distinct responses between the two bacterial species to chloramphenicol. In the case of *S. aureus*, complete inhibition of growth was observed at a concentration of 3.125 µg mL^{-1}, as indicated by the absence of color change and retention of the blue color. In contrast, *E. coli* exhibited a higher level of tolerance, requiring a concentration of 6.25 µg mL^{-1} to reach the MIC.

The observed differences in susceptibility between *S. aureus* and *E. coli* are likely related to the structural and physiological characteristics that distinguish Gram-positive and Gram-negative bacteria. *Staphylococcus aureus*, as a Gram-positive organism, possesses a thick but relatively permeable peptidoglycan layer that facilitates the penetration of hydrophobic antibiotics such as chloramphenicol. In contrast, *E. coli* has

an outer membrane (OM) that acts as a selective permeability barrier, reducing antibiotic influx and enhancing efflux mechanisms, thereby limiting intracellular drug accumulation (Gauba and Rahman, 2023). In addition, Gram-negative bacteria frequently exhibit other resistance strategies, including enzymatic drug inactivation or modification, which further compromises antibiotic efficacy (Gauba and Rahman, 2023). Collectively, these mechanisms increase the concentration of antibiotic required to achieve bacterial inhibition and, in some cases, necessitate the use of alternative or combined antimicrobial strategies (Ruppé, Woerther and Barbier, 2015; Baker et al., 2018; Urban-Chmiel et al., 2022).

The MIC values obtained in this study are consistent with previous reports. Zia et al. (2023) demonstrated that *E. coli* generally exhibits higher resistance to several antibiotics, including ampicillin and gentamicin, when compared to *S. aureus*. Additionally, Li et al. (2024) reported the presence of chloramphenicol-sensitive *E. coli* strains with complete inhibition at concentrations as low as 1 μg mL^{-1} when chloramphenicol conjugates were used. However, substantial variability in chloramphenicol susceptibility has been reported worldwide. For instance, Gil-Gil and Berryhill (2025) reported MIC values of 6.25 μg mL^{-1} for *E. coli* MG1655 and 8 μg mL^{-1} for *S. aureus*. Conversely, highly resistant *E. coli* isolates with MIC values up to 32 μg mL^{-1} have also been documented (Puangseree et al., 2024).

These comparisons highlight the inherent variability of MIC values, which depends not only on bacterial species but also on strain origin, genetic background, and the presence of specific resistance mechanisms.

2.4.8.2 Rhamnolipid crude extract MIC value

The determination of the minimum inhibitory concentration (MIC) is a fundamental procedure in microbiology, as it defines the lowest concentration of an antimicrobial agent capable of preventing visible microbial growth under in vitro conditions (Kadeřábková, Mahmood and Mavridou, 2024). This parameter not only enables quantitative comparison among different antimicrobial compounds across microbial species and experimental conditions (Wiegand, Hilpert and Hancock, 2008; Wen et al., 2016), but also plays a critical role in the identification and selection of new therapeutic strategies (Andrews, 2001; Kowalska-Krochmal and Dudek-Wicher, 2021).

Furthermore, MIC determination is essential for evaluating novel antimicrobial candidates, whether as individual compounds, combinations of existing drugs, or new bioactive products with antimicrobial potential (Gyawali and Ibrahim, 2014; Kamble, Sanghvi and Pardesi, 2022; Choudhury, Haq and Kalamdhad, 2023).

In the clinical context, MIC values are particularly important because they directly support informed decision-making in the treatment of infectious diseases. MIC testing guides antibiotic selection, contributes to the establishment of susceptibility breakpoints, and is widely integrated into routine clinical microbiology diagnostics. Accurate MIC determination is therefore essential not only to prevent therapeutic failure, but also to reduce the selective pressure that drives antimicrobial resistance and to improve overall patient recovery outcomes (Kowalska-Krochmal and Dudek-Wicher, 2021).

In the present study, MIC determination was applied to evaluate the antimicrobial potential of rhamnolipids produced by the three strains (*Pseudomonas aeruginosa* LBBMA 58, LBBMA 79, and LBBMA 92) against *Escherichia coli* and *Staphylococcus aureus*. The results demonstrated a clear dose–response relationship between rhamnolipid concentration and bacterial growth inhibition. As rhamnolipid concentration increased, bacterial growth was progressively suppressed for both species.

For *E. coli*, high levels of inhibition were observed at the highest tested concentration (1,000 μg mL^{-1}), reaching 90.9% for RL-58, 86.3% for RL-79, and 88.7% for RL-92. According to the criterion proposed by Campbell (2011), compounds can be considered growth-inhibitory when bacterial survival is below 10% relative to the positive control. Based on this definition, inhibition values above 90% were interpreted as reaching the MIC (Figures 11 and 12). Under these conditions, only RL-58 reached the MIC against *E. coli*. Nevertheless, RL-79 and RL-92 exhibited strong inhibitory effects, approaching the MIC threshold, suggesting that their MIC values are higher than 1,000 μg mL^{-1}.

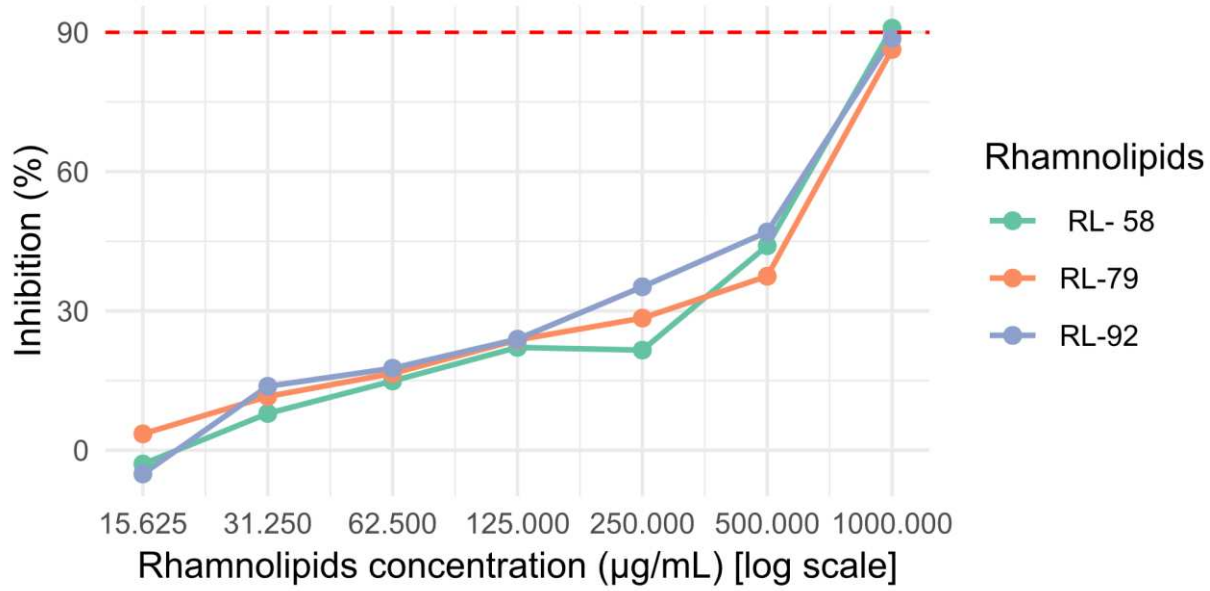
A similar pattern was observed for *S. aureus*. RL-58 achieved the MIC at 1,000 μg mL^{-1}, with a growth inhibition of 98.2% (Figure 12). In contrast, RL-79 and RL-92

showed lower inhibitory effects at the same concentration, with inhibition values of 78.7% and 57.5%, respectively.

Interestingly, inhibitory effects were also detected at lower rhamnolipid concentrations. At 15.63 μg mL^{-1}, growth inhibition against *S. aureus* reached 35.9% for RL-58, 26.35% for RL-79, and 19.95% for RL-92. In contrast, inhibition of *E. coli* at this concentration was minimal, ranging from 0% to 5%. However, as rhamnolipid concentrations increased beyond 31.25 μg mL^{-1}, *E. coli* exhibited a sharp increase in sensitivity, with inhibition values approaching 90% for all three rhamnolipid extracts at higher doses (Figure 11).

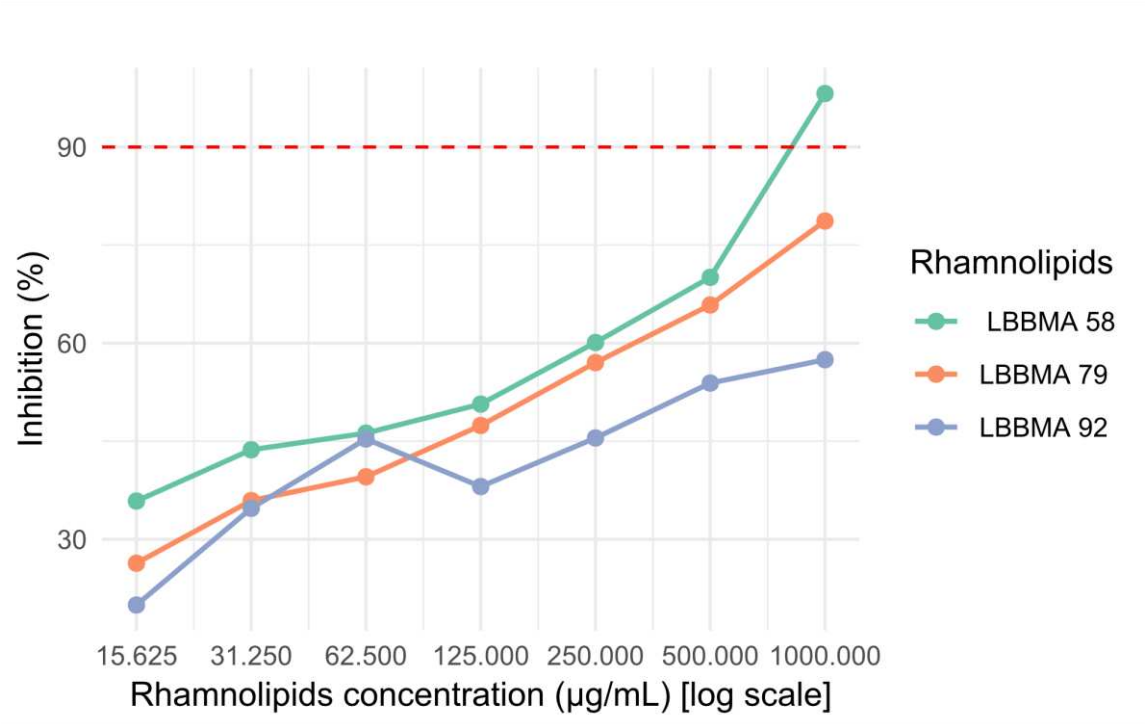
In contrast, *S. aureus* displayed a different response pattern. At lower rhamnolipid concentrations, *S. aureus* appeared more sensitive than *E. coli*. However, at higher concentrations, *S. aureus* exhibited greater tolerance, particularly toward RL-79 and RL-92. This differential behavior may be partially explained by the antimicrobial susceptibility profiles shown in Tables 5 and 6. While *E. coli* did not exhibit resistance to any of the tested antibiotics, *S. aureus* showed intermediate resistance to azithromycin and resistance to penicillin. These findings suggest that *S. aureus* may possess intrinsic or acquired resistance mechanisms that reduce susceptibility to certain antimicrobial agents, including specific rhamnolipid extracts such as RL-79 and RL-92.

Figure 11. Inhibition of *Escherichia coli* growth in presence of rhamnolipids crude extract in several concentrations.



The inhibition percentage (%) of *E. coli ECC* was evaluated across a concentration range of 1000 - 15.6 µg mL^{-1} (log scale). The red dashed line denotes the 90 % of inhibition. This boundary was used to establish the minimum inhibitory concentration (MIC).

Figure 12. Inhibition of *S. aureus* growth in presence of RL-58, RL-79 and RL-92 in several concentrations.



The inhibition percentage (%) of *S. aureus SAC* was evaluated across a concentration range of 1000 - 15.6 µg/mL (log scale). The red dashed line denotes the 90 % of inhibition that was used as the boundary to determine the minimum inhibitory concentration (MIC).

According to Duarte et al. (2007), MIC values allow antimicrobial compounds to be classified into three categories based on their inhibitory strength. Compounds with MIC values ≤ 500 µg mL^{-1} are considered strong inhibitors of bacterial growth. MIC values between > 600 and ≤ 1500 µg mL^{-1} indicate moderate inhibitory activity, whereas MIC values > 1500 µg mL^{-1} are classified as weak inhibitory activity. Based on this classification, the rhamnolipid produced by strain LBBMA 58 (RL-58) against both *S. aureus* and *E. coli* can be classified as a compound with moderate inhibitory activity. For RL-79 and RL-92, the MIC values against *E. coli* appear to be close to 1000 µg mL^{-1}, suggesting moderate inhibitory potential as well. However, in the case of *S. aureus*, the rhamnolipid produced by strain LBBMA 92 required concentrations exceeding 1000 µg mL^{-1} to achieve growth inhibition ≥ 90%, indicating a lower antimicrobial effectiveness under the tested conditions. Consequently, among the three strains evaluated, *P. aeruginosa* LBBMA 58 exhibited the strongest and most consistent inhibitory effect against both *E. coli* and *S. aureus*.

It is important to emphasize that the concentrations tested in this study did not exceed 1000 μg mL^{-1}, which was deliberately selected as a conservative upper limit. Several studies have reported that higher rhamnolipid concentrations may induce cytotoxic and hemolytic effects, thereby limiting their safe application (Haba et al., 2014). For example, Thakur et al. (2021) demonstrated that rhamnolipid concentrations above 3000 μg mL^{-1} caused significant cellular damage, while Touabi et al. (2025) reported reduced cell viability at concentrations ranging from 60 to 1000 μg mL^{-1}.
These findings support the decision to restrict the maximum rhamnolipid concentration to 1000 μg mL^{-1} in the present study. This approach ensures that antimicrobial activity is evaluated within a biologically relevant and safety-conscious range, increasing the translational potential of the results and reinforcing the feasibility of rhamnolipids as antimicrobial agents that could be applied without compromising cellular integrity or patient safety.

2.4.9 Minimal inhibitory concentration (MIC) of chloramphenicol combined with rhamnolipids crude extract

Chloramphenicol antibiotics, which include chloramphenicol (CLO) and its analogs thiamphenicol and florfenicol, are broad-spectrum antimicrobials that have historically been used due to their activity against a wide range of Gram-positive and Gram-negative bacteria, demonstrating effectiveness in both veterinary and human medicine (Lin et al., 2022). Chloramphenicol exerts its antibacterial activity by inhibiting microbial protein synthesis through binding to the 50S ribosomal subunit (Sarmah, Meyer and Boxall, 2006; Chatzitakis et al., 2008).

Because of its low cost, high availability, and broad-spectrum efficacy, CLO was widely used during the 1950s for the treatment of infectious diseases in both humans and domestic animals (Sarmah, Meyer and Boxall, 2006; Chatzitakis et al., 2008). Despite these advantages, the clinical use of chloramphenicol has been severely restricted due to its association with dose-independent bone marrow suppression. Currently, its clinical use is restricted to rare and severe human infections, such as life-threatening meningitis, when no safer alternatives are available (McCurdy and Gieschen, 1963; Sarmah, Meyer and Boxall, 2006).

Despite these limitations, chloramphenicol remains a valuable in vitro comparator and a potential candidate for combination therapies. In such strategies, synergistic interactions could allow dose reduction and consequently mitigate toxicity concerns.

However, in contrast to this expectation, the results of the present study revealed antagonistic rather than synergistic interactions (Table 7 when chloramphenicol was combined with rhamnolipids produced by strains LBBMA 79 (RL-79) and LBBMA 92 (RL-92) at the tested concentrations. To calculate the Fractional Inhibitory Concentration Index (FICI), MIC values of both chloramphenicol and rhamnolipids were used. According to standard FICI interpretation criteria (Table 7), the combinations of CLO with RL-79 and RL-92 exhibited clear antagonism against both *Escherichia coli* and *Staphylococcus aureus*, with FICI values ranging from 4.0 to 4.25 for *E. coli* and from 8.0 to 15.0 for *S. aureus*.

In the case of RL-58, the interaction with chloramphenicol resulted in an indifferent effect against *E. coli*, whereas an antagonistic interaction was observed against *S. aureus*. These findings indicate that rhamnolipid–antibiotic interactions are highly strain-dependent and may compromise antimicrobial efficacy when combined indiscriminately.

Table 7. Minimum inhibitory concentration (MIC) values of chloramphenicol (CLO) in combination with rhamnolipid crude extracts (RL) produced by *Pseudomonas* strains LBBMA 58 (RL-58), LBBMA 79 (RL-79), and LBBMA 92 (RL-92) against *Escherichia coli* and *Staphylococcus aureus*.

Organism	Combination	MIC CLO (μg mL^{-1})	MIC RL (μg mL^{-1})	FICI	Interpretation
E. coli	CLO + RL-58	12.5	3.91	2,00	Indifferent
E. coli	CLO + RL-79	25	3.91	>4.00 and <4.25	Antagonism
E. coli	CLO + RL-92	25	250	>4.00 and	Antagonism

				<4.25	
S. aureus	CLO + RL-58	25	250	8.25	Antagonism
S. aureus	CLO + RL-79	25	250	>8.00 and <8.06	Antagonism
S. aureus	CLO + RL-92	25	62.5	>15.00 and <15,40	Antagonism

Several studies have reported the potential of chloramphenicol when used in combination therapies with other antibiotics, demonstrating synergistic interactions in specific contexts (Rahal and Simberkoff, 1979; Danesh et al., 2016). Conversely, numerous reports have shown that chloramphenicol can exert antagonistic effects when combined with other antimicrobial agents, particularly β-lactam antibiotics such as ampicillin, penicillin, cefotaxime, and ceftriaxone (Weeks, Mason and Baker, 1981; Asmar, Prainito and Dajani, 1988; Acar, 2000). This apparent duality underscores the complexity of antibiotic–antibiotic and antibiotic–molecule interactions, which depend on multiple factors, including the mechanisms of action of the compounds involved, their physicochemical compatibility, the physiological state of the bacterial cell, and the experimental conditions under which the interaction is assessed.

The antagonistic effect observed in the present study is not yet fully understood; however, it may be explained by several mechanisms that could occur simultaneously. Chloramphenicol is a bacteriostatic antibiotic that inhibits protein synthesis by binding to the 50S ribosomal subunit (Sanga and Kharel, 2023). In contrast, rhamnolipids are amphiphilic biosurfactants that primarily interact with bacterial membranes, altering membrane permeability and, in some cases, influencing efflux pump activity (Poole, 2005). Such membrane perturbations may reduce the intracellular accumulation of hydrophobic molecules, including chloramphenicol.

Additionally, rhamnolipids are known to form micelles capable of encapsulating or transporting hydrophobic compounds (Gdaniec et al., 2022). Given the relatively hydrophobic nature of chloramphenicol, it is plausible that rhamnolipid micelles may partially entrap the antibiotic, thereby reducing the fraction of free, bioavailable chloramphenicol and diminishing its antimicrobial efficacy. This physicochemical

interaction between rhamnolipids and chloramphenicol in aqueous systems has been previously documented in the context of antibiotic removal from contaminated water (Lin et al., 2022), supporting the hypothesis that sequestration can occur.

The differential behavior observed with RL-58 against E. coli (indifference) compared to RL-79 and RL-92 (antagonism) warrants further consideration. RL-58 not only maintained its own antimicrobial activity (with its MIC dropping to 3.91 µg mL^{-1} in combination) but also increased the MIC of chloramphenicol. This dual effect suggests a distinct mechanistic profile for this extract. One hypothesis is that the specific congener composition of RL-58 (Figure 6) may confer a higher propensity for micelle formation, potentially sequestering chloramphenicol more efficiently and thereby reducing its bioavailability. Simultaneously, this same composition could render RL-58 intrinsically more toxic to bacterial cells, explaining its sustained inhibitory activity even in the presence of the antibiotic. The balance between mono- and di-rhamnolipids is known to influence biological activity; for instance, a higher proportion of mono-rhamnolipids can enhance the ability to interact with and disrupt bacterial membranes (Zhao et al., 2022). Therefore, RL-58 may represent a mixture in which the dual functionality of membrane disruption and antibiotic sequestration is particularly pronounced, leading to the observed indifferent interaction.

Together, these physicochemical and physiological interactions provide a plausible explanation for the antagonistic effects observed between chloramphenicol and rhamnolipids produced by strains LBBMA 79 and LBBMA 92 in both bacterial species, as well as with RL-58 in the case of Staphylococcus aureus. Nevertheless, targeted mechanistic studies will be required to elucidate the relative contribution of these processes and to fully clarify the molecular basis of the observed antagonism.

2.4.10 Suppression of fungal growth assess

The evaluation of fungal growth in the presence of crude rhamnolipid extracts produced by strains LBBMA 58, LBBMA 79, and LBBMA 92 revealed an unexpected pattern when compared with previous reports. Rather than inhibiting fungal development, high rhamnolipid concentrations stimulated fungal growth. As shown in Figure 13, all three rhamnolipid extracts promoted the growth of *Corynespora*

cassiicola across several concentrations, with growth values reaching approximately 220–240% relative to the control (Table 8). Under the experimental conditions employed in this study, these results indicate that rhamnolipids did not act as antifungal agents against the target organism. Instead, they functioned as growth-promoting factors.

Although all three strains exhibited a similar overall trend, differences in the magnitude of stimulation were observed. The rhamnolipid extract produced by strain LBBMA 92 (RL-92) showed the strongest effect, reaching growth values of approximately 240% at 2000 µg mL^{-1} (Figure 13). In contrast, RL-79 induced the lowest stimulation, with growth values around 180%, while RL-58 exhibited an intermediate effect (~220%). At lower concentrations (31.25–125 µg mL^{-1}), the response was more heterogeneous. Specifically, RL-79 and RL-58 caused a slight reduction in fungal growth (up to ~6.5% relative to the control), whereas RL-92 consistently stimulated fungal growth even at the lowest concentrations tested.

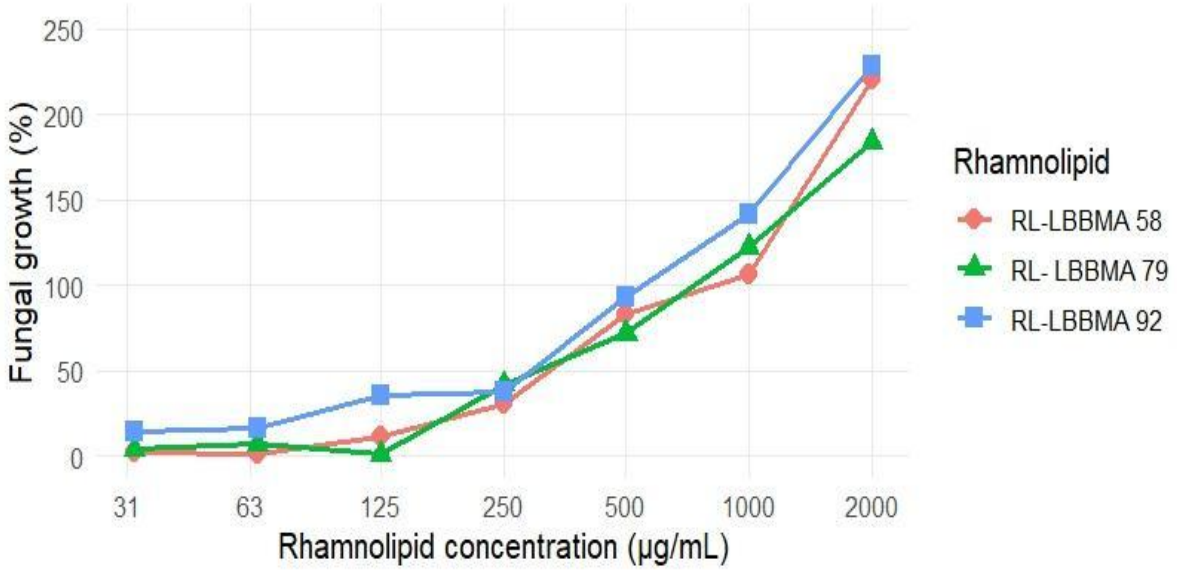
Table 8. Fungal growth of *Corynespora cassiicola* in the presence of different concentrations of rhamnolipids crude extract (RL) produced by *Pseudomonas* strains LBBMA 58 (RL- 58), LBBMA 79 (RL-79), and LBBMA 92 (RL-92).

-	*Corynespora cassiicola* growth in presence of rhamnolipids		
Concentration (ug mL^{-1})	RL- 58	RL- 79	RL- 92
2000	3.48 ± 0.26	3.11 ± 0.67	3.56 ± 0.17
1000	2.34 ± 0.11	2.50 ±0.90	2.69 ± 0.54
500	2.10 ± 0.50	1.99 ± 0.77	2.21 ± 0.59
250	1.58 ± 0.15	1.69 ± 0.51	1.61 ±0.27
125	1.38 ± 0.07	1.28 ± 0.65	1.63 ± 0.31
62.5	1.28 ± 0.17	1.34 ± 0.25	1.43 ± 0.13

31.25	1.29 ± 0.04	1.31 ± 0.24	1.41 ± 0.11
Blank	0.012 ± 0.01	Positive control	1.37 ± 0.09

Values represent mean ± standard deviation of optical density (OD) readings at 630 nm, indicating the relative fungal biomass after incubation with each rhamnolipid concentration.

Figure 13. Effect of rhamnolipids crude extract on the growth of *Corynespora cassiicola*.



The graph shows the percentage of fungal growth in response to the increase of the concentrations of rhamnolipids (RL)

Nevertheless, antifungal activity is generally considered strong or biologically relevant only when growth inhibition exceeds 50% compared to the control, which is commonly used as a threshold criterion (Floyd et al., 2024). In contrast, inhibition values below 50% are typically regarded as non-meaningful antifungal activity. By this metric, none of the rhamnolipid extracts tested in this study would be classified as possessing

meaningful antifungal activity against C. cassiicola. However, framing the results solely in terms of "non-meaningful inhibition" fails to capture their true biological significance. The observed response was not merely an absence of inhibition, but rather a consistent and, in some cases, pronounced stimulation of fungal growth, reaching up to 240% of the control at the highest concentrations tested. This qualitative difference - moving from inhibition to promotion - is far more critical than a quantitative failure to meet a 50% inhibition threshold. It fundamentally alters the risk assessment, transforming rhamnolipids from potential antifungal agents into compounds that could inadvertently exacerbate fungal diseases in agricultural or clinical settings. This finding underscores the necessity of evaluating not only the magnitude but also the direction of the biological response when assessing the potential of new antimicrobial compounds.

In the case of strains LBBMA 58 and LBBMA 79, the observed results suggest a possible hormetic response, in which low concentrations of biosurfactants induce mild stress leading to partial growth inhibition, whereas intermediate or higher concentrations may be metabolized by the fungus as an additional carbon source. This growth-promoting effect suggests that C. cassiicola may be capable of metabolizing rhamnolipids to support its development. This hypothesis is consistent with the known ecological and metabolic versatility of this fungus. C. cassiicola is a necrotrophic pathogen with a broad host range, and its genome encodes a diverse array of carbohydrate-active enzymes (CAZymes) and other hydrolytic enzymes that enable it to degrade plant cell walls and utilize a wide variety of organic substrates (Lopez et al., 2018). Furthermore, metabolic phenotyping studies have demonstrated that C. cassiicola can metabolize approximately 89% of tested carbon sources, underscoring its remarkable nutritional adaptability (Lopez et al., 2018). Given that rhamnolipids are glycolipids—comprising both a sugar head and a lipid tail—they may be susceptible to cleavage by these fungal hydrolases, releasing assimilable compounds that support fungal growth. Because rhamnolipids are glycolipids, their utilization as substrates at relatively low concentrations is structurally plausible and may explain the enhanced biomass production observed at higher concentrations.

In addition, the composition of the culture medium used for rhamnolipid production should be taken into account. This medium contains glucose and other nutrients, and trace amounts of these components may remain in the crude extracts despite

downstream processing. The presence of residual sugars and other readily assimilable compounds may further facilitate fungal growth under in vitro conditions, particularly when combined with the assay medium. The interaction between rhamnolipids, fatty acid fractions, and medium-derived carbon sources may therefore create favorable conditions for fungal development, partially masking or counteracting any intrinsic antifungal activity of the biosurfactant.

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The selection of test concentrations was based on previous studies reported in the literature. A dose-dependent analysis conducted by Deepika, Ramu Sridhar and Bramhachari (2015) demonstrated that the application of rhamnolipids at 200 μg mL^{-1} in tomato plants infected with the pathogenic fungus *Fusarium oxysporum* resulted in complete disease recovery (100%). According to the authors, a concentration of 100 μg mL^{-1} reduced disease severity by approximately 50%. Similarly, Mohama Shukri et al. (2025) reported complete inhibition (100%) of the plant pathogenic fungus *Rhizoctonia solani* at a concentration of 10,000 μg mL^{-1}. In a separate study, Szczepaniak et al. (2015) found that rhamnolipids at 600 mg L^{-1} significantly inhibited plant root growth (69% inhibition). Furthermore, when rhamnolipids were directly compared with synthetic surfactants at the same concentration (600 mg L^{-1}), the authors observed that the biosurfactant exhibited toxicity levels comparable to those induced by the widely used synthetic surfactant Tween 80.

Additionally, Gidudu et al. (2022) demonstrated significant phytotoxic effects when purified biosurfactants were applied to three plant species (*Pisum sativum*, *Phaseolus vulgaris*, and *Brassica napus*). According to the authors, root elongation was negatively affected at all tested doses, with the concentration of 100 μg mL^{-1} producing the most pronounced inhibitory effect. Collectively, these studies indicate that although

increasing rhamnolipid concentration may raise the likelihood of fungal growth inhibition, it simultaneously increases the risk of inducing undesirable phytotoxic effects in plants.

Although rhamnolipids are broadly recognized as antimicrobial agents, the results of the present study emphasize that their inhibitory interaction with fungi is not guaranteed. From a biotechnological perspective, this highlights the importance of careful evaluation before promoting rhamnolipids as antifungal agents, since, as observed here, their application may in some cases stimulate rather than suppress fungal growth. This dual behavior suggests potential alternative applications, such as enhancers of fungal biomass production or secondary metabolite synthesis. However, these findings also caution against the indiscriminate use of rhamnolipids as antifungal compounds in agricultural or medical contexts.

2.5 Conclusions

In conclusion, the isolates LBBMA 58, LBBMA 79, and LBBMA 92 were assigned to the genus *Pseudomonas* based on partial 16S rRNA, *rpoD*, and *gyrB* gene sequences. Their phylogenetic placement indicates a close relationship with *Pseudomonas aeruginosa*; however, additional analyses, such as whole-genome sequencing, are required to definitively confirm their taxonomic identity. Among the three isolates, LBBMA 92 achieved the highest rhamnolipid production yield, whereas LBBMA 58 exhibited the lowest production levels. All crude extracts effectively reduced surface tension to values below 30 mN m^{-1} and displayed substrate-dependent emulsifying activity, with petroleum being the most responsive substrate. LC–MS/MS analysis confirmed the presence of rhamnolipids in all samples and revealed a predominance of di-rhamnolipids, consistent with profiles commonly reported for *Pseudomonas* strains and commercial rhamnolipid standards.

From a biological perspective, rhamnolipids produced by all three strains were able to inhibit bacterial growth in both *Escherichia coli* and *Staphylococcus aureus*. However, when combined with chloramphenicol, rhamnolipids predominantly exhibited antagonistic interactions, with only one indifferent interaction observed (RL-58 against *E. coli*). In contrast, rhamnolipids consistently stimulated the growth of *Corynespora cassiicola*, rather than exerting antifungal activity. Collectively, these findings demonstrate that rhamnolipid activity is strongly influenced by the producing strain,

concentration, and target microorganism. Consequently, the successful application of rhamnolipids—whether as antimicrobial agents or in other biotechnological contexts—requires a comprehensive evaluation that considers the intrinsic characteristics of the rhamnolipid mixture and its interactions with the biological and environmental context. Finally, although the critical micelle concentration (CMC) is often regarded as a key physicochemical parameter for biosurfactant characterization, this study demonstrates that CMC alone is insufficient to predict functional performance. Despite the excellent surface tension–reducing capacity of the rhamnolipids produced, this property did not consistently correlate with antimicrobial or antifungal activity. Therefore, a thorough physicochemical and biological characterization is essential to ensure the safe, effective, and context-appropriate use of rhamnolipids across diverse fields, including agriculture, medicine, veterinary science, and environmental management.

REFERENCES

Abdel-Mawgoud, A.M., Lépine, F. and Déziel, E. (2010) “Rhamnolipids: Diversity of structures, microbial origins and roles,” *Applied Microbiology and Biotechnology*, 86(5), pp. 1323–1336. Available at: https://doi.org/10.1007/S00253-010-2498-2,.

Abooali, D. and Soleimani, R. (2023) “Structure-based modeling of critical micelle concentration (CMC) of anionic surfactants in brine using intelligent methods,” *Scientific Reports*, 13(1), pp. 1–16. Available at: https://doi.org/10.1038/S41598-023-40466-1;SUBJMETA.

Acar, J.F. (2000) “Antibiotic synergy and antagonism,” *The Medical clinics of North America*, 84(6), pp. 1391–1406. Available at: https://doi.org/10.1016/S0025-7125(05)70294-7.

Albasri, H.M. *et al.* (2024) “Production and characterization of rhamnolipid biosurfactant from thermophilic Geobacillus stearothermophilus bacterium isolated from Uhud mountain,” *Frontiers in Microbiology*, 15, p. 1358175. Available at: https://doi.org/10.3389/FMICB.2024.1358175.

Al-Shimmary, S.M.H. *et al.* (2021) “Phylogeny Analysis of gyrB Gene and 16S rRNA Genes of *Pseudomonas aeruginosa* Isolated from Iraqi Patients.,” *Research Journal of Pharmacy and Technology*, 14(5), pp. 2517–2521. Available at: https://doi.org/10.52711/0974-360X.2021.00443.

Alwadani, N. and Fatehi, P. (2018) “Synthetic and lignin-based surfactants: Challenges and opportunities,” *Carbon Resources Conversion*, 1(2), pp. 126–138. Available at: https://doi.org/10.1016/J.CRCON.2018.07.006.

Ambaye, T.G. *et al.* (2022) “Insights into rhamnolipid amendment towards enhancing microbial electrochemical treatment of petroleum hydrocarbon contaminated soil,” *Chemosphere*, 307, p. 136126. Available at: https://doi.org/10.1016/J.CHEMOSPHERE.2022.136126.

Andrews, J.M. (2001) “Determination of minimum inhibitory concentrations,” *The Journal of antimicrobial chemotherapy*, 48 Suppl 1(SUPPL. 1), pp. 5–16. Available at: https://doi.org/10.1093/JAC/48.SUPPL_1.5.

Araujo, J.S. de *et al.* (2020) “Production of Rhamnolipids by *Pseudomonas aeruginosa* AP029-GLVIIA and Application on Bioremediation and as a Fungicide,” *Biosciences Biotechnology Research Asia*, 17(03), pp. 467–477. Available at: https://doi.org/10.13005/bbra/2850.

Asmar, B.I., Prainito, M. and Dajani, A.S. (1988) “Antagonistic effect of chloramphenicol in combination with cefotaxime or ceftriaxone,” *Antimicrobial Agents and Chemotherapy*, 32(9), p. 1375. Available at: https://doi.org/10.1128/AAC.32.9.1375.

Bahia, F.M. *et al.* (2018) “Rhamnolipids production from sucrose by engineered Saccharomyces cerevisiae,” *Scientific Reports 2018 8:1*, 8(1), pp. 1–10. Available at: https://doi.org/10.1038/s41598-018-21230-2.

Banat, I.M. et al. (2010) “Microbial biosurfactants production, applications and future potential,” Applied microbiology and biotechnology, 87(2), pp. 427–444. Available at: https://doi.org/10.1007/S00253-010-2589-0.

Bjerk, T.R. et al. (2021) “Biosurfactants: Properties and Applications in Drug Delivery, Biotechnology and Ecotoxicology,” Bioengineering, 8(8), p. 115. Available at: https://doi.org/10.3390/BIOENGINEERING8080115.

Caiazza, N.C., Shanks, R.M.Q. and O’Toole, G.A. (2005) “Rhamnolipids modulate swarming motility patterns of *Pseudomonas aeruginosa*,” *Journal of bacteriology*, 187(21), pp. 7351–7361. Available at: https://doi.org/10.1128/JB.187.21.7351-7361.2005.

Campbell, J. (2011) “High-throughput assessment of bacterial growth inhibition by optical density measurements,” *Current protocols in chemical biology*, 3(3), p. 100115. Available at: https://doi.org/10.1002/9780470559277.CH100115.

Cerqueira dos Santos, S. *et al.* (2024) “Production and characterization of rhamnolipids by *Pseudomonas aeruginosa* isolated in the Amazon region, and potential antiviral, antitumor, and antimicrobial activity,” *Scientific Reports*, 14(1), pp. 1–13. Available at: https://doi.org/10.1038/S41598-024-54828-W;SUBJMETA.

Chatzitakis, A. *et al.* (2008) “Photocatalytic degradation and drug activity reduction of Chloramphenicol,” *Water Research*, 42(1–2), pp. 386–394. Available at: https://doi.org/10.1016/J.WATRES.2007.07.030.

Chen, W.C., Juang, R.S. and Wei, Y.H. (2015) “Applications of a lipopeptide biosurfactant, surfactin, produced by microorganisms,” Biochemical Engineering Journal, 103, pp. 158–169. Available at: https://doi.org/10.1016/J.BEJ.2015.07.009.

Choudhury, S.P., Haq, I. and Kalamdhad, A.S. (2023) “Unleashing synergistic potential of microbially enhanced anaerobic co-digestion of petroleum refinery biosludge and yard waste: Impact of nutrient balance and microbial diversity,” *Journal of Hazardous Materials*, 460. Available at: https://doi.org/10.1016/j.jhazmat.2023.132361.

Christova, N. *et al.* (2011) “Chemical characterization and physical and biological activities of rhamnolipids produced by *Pseudomonas aeruginosa* BN10,” *Zeitschrift fur Naturforschung - Section C Journal of Biosciences*, 66 C(7–8), pp. 394–405. Available at: https://doi.org/10.1515/ZNC-2011-7-811,.

Curran, B. *et al.* (2004) “Development of a Multilocus Sequence Typing Scheme for the Opportunistic Pathogen *Pseudomonas aeruginosa*,” *Journal of Clinical Microbiology*, 42(12), p. 5644. Available at: https://doi.org/10.1128/JCM.42.12.5644-5649.2004.

Danesh, A. *et al.* (2016) “Synergistic effect of haloduracin and chloramphenicol against clinically important Gram-positive bacteria,” *Biotechnology Reports*, 13, p. 37. Available at: https://doi.org/10.1016/J.BTRE.2016.12.008.

Davey, M.E., Caiazza, N.C. and O'Toole, G.A. (2003) "Rhamnolipid Surfactant Production Affects Biofilm Architecture in *Pseudomonas aeruginosa* PAO1," *Journal of Bacteriology*, 185(3), p. 1027. Available at: https://doi.org/10.1128/JB.185.3.1027-1036.2003.

Duarte, M.C.T. *et al.* (2007) "Activity of essential oils from Brazilian medicinal plants on Escherichia coli," *Journal of Ethnopharmacology*, 111(2), pp. 197–201. Available at: https://doi.org/10.1016/j.jep.2006.11.034.

Effendi, I. *et al.* (2017) "Detergent Disposal into Our Environmentand Its Impact on Marine Microbes," *IOP Conference Series: Earth and Environmental Science*, 97(1). Available at: https://doi.org/10.1088/1755-1315/97/1/012030.

Elarbi, F.M. *et al.* (2020) "Determination of CMC and interfacial properties of anionic (SDS) and cationic (CPB) surfactants in aqueous solutions," *American Journal of Engineering Research (AJER)*, 9(8), pp. 118–126. Available at: www.ajer.org

El-Housseiny, G.S. *et al.* (2020) "Structural and Physicochemical Characterization of Rhamnolipids produced by *Pseudomonas aeruginosa* P6," *AMB Express*, 10(1), pp. 1–12. Available at: https://doi.org/10.1186/S13568-020-01141-0/FIGURES/5.

Elshikh, M. *et al.* (2016) "Resazurin-based 96-well plate microdilution method for the determination of minimum inhibitory concentration of biosurfactants," *Biotechnology Letters*, 38(6), p. 1015. Available at: https://doi.org/10.1007/S10529-016-2079-2.

Gauba, A. and Rahman, K.M. (2023) "Evaluation of Antibiotic Resistance Mechanisms in Gram-Negative Bacteria," *Antibiotics 2023, Vol. 12, Page 1590*, 12(11), p. 1590. Available at: https://doi.org/10.3390/ANTIBIOTICS12111590.

Gdaniec, B.G. *et al.* (2022) "*Pseudomonas aeruginosa* rhamnolipid micelles deliver toxic metabolites and antibiotics into Staphylococcus aureus," *iScience*, 25(1), p. 103669. Available at: https://doi.org/10.1016/J.ISCI.2021.103669.

Gil-Gil, T. and Berryhill, B.A. (2025) “Antibiotic killing of drug-induced bacteriostatic cells,” *Antimicrobial Agents and Chemotherapy*, 69(5). Available at: https://doi.org/10.1128/AAC.00156-25.

Girard, L. *et al.* (2020) “Reliable identification of environmental *Pseudomonas* isolates using the rpod gene,” *Microorganisms*, 8(8), pp. 1–13. Available at: https://doi.org/10.3390/MICROORGANISMS8081166

Goveas, L.C. *et al.* (2024) “Rhamnolipid assisted degradation of petroleum crude oil by indigenous *Pseudomonas* sp. WDE11 in seawater,” *Journal of Environmental Chemical Engineering*, 12(1), p. 111693. Available at: https://doi.org/10.1016/J.JECE.2023.111693.

Gyawali, R. and Ibrahim, S.A. (2014) “Natural products as antimicrobial agents,” *Food Control*, 46, pp. 412–429. Available at: https://doi.org/10.1016/J.FOODCONT.2014.05.047.

Haba, E. *et al.* (2014) “Complex rhamnolipid mixture characterization and its influence on DPPC bilayer organization,” *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1838(3), pp. 776–783. Available at: https://doi.org/10.1016/J.BBAMEM.2013.11.004.

Holtappels, M. *et al.* (2018) “Antifungal activity of oleylphosphocholine on in Vitro and in Vivo Candida Albicans biofilms,” *Antimicrobial Agents and Chemotherapy*, 62(1). Available at: https://doi.org/10.1128/AAC.01767-17/SUPPL_FILE/ZAC001186781SM2.AVI.

Hošková, M. *et al.* (2015) “Structural and physiochemical characterization of rhamnolipids produced by Acinetobacter calcoaceticus, Enterobacter asburiae and *Pseudomonas aeruginosa* in single strain and mixed cultures,” *Journal of Biotechnology*, 193, pp. 45–51. Available at: https://doi.org/10.1016/J.JBIOTEC.2014.11.014.

Hosseini, S. *et al.* (2024) “Molecular identification of rhamnolipids produced by *Pseudomonas* oryzihabitans during biodegradation of crude oil,” *Frontiers in Microbiology*, 15. Available at: https://doi.org/10.3389/FMICB.2024.1459112

Hsu, C.Y. *et al.* (2025) “Biosurfactants: Properties, applications and emerging trends,” *South African Journal of Chemical Engineering*, 53, pp. 21–39. Available at: https://doi.org/10.1016/J.SAJCE.2025.04.002.

Johnson, P. *et al.* (2021) “Effect of synthetic surfactants on the environment and the potential for substitution by biosurfactants,” *Advances in Colloid and Interface Science*, 288, p. 102340. Available at: https://doi.org/10.1016/J.CIS.2020.102340.

Kadeřábková, N., Mahmood, A.J.S. and Mavridou, D.A.I. (2024) “Antibiotic susceptibility testing using minimum inhibitory concentration (MIC) assays,” *npj Antimicrobials and Resistance*, 2(1), pp. 1–9. Available at: https://doi.org/10.1038/S44259-024-00051-6;SUBJMETA.

Kamble, E., Sanghvi, P. and Pardesi, K. (2022) “Synergistic effect of antibiotic combinations on Staphylococcus aureus biofilms and their persister cell populations,” *Biofilm*, 4. Available at: https://doi.org/10.1016/j.bioflm.2022.100068.

Kowalska-Krochmal, B. and Dudek-Wicher, R. (2021) “The Minimum Inhibitory Concentration of Antibiotics: Methods, Interpretation, Clinical Relevance,” *Pathogens*, 10(2), p. 165. Available at: https://doi.org/10.3390/PATHOGENS10020165.

Li, D. *et al.* (2022) “Emulsifying Properties of Rhamnolipids and Their In Vitro Antifungal Activity against Plant Pathogenic Fungi,” *Molecules*, 27(22). Available at: https://doi.org/10.3390/MOLECULES27227746.

Li, H. *et al.* (2023) “Quantitative analysis of biosurfactants in water samples by a modified oil spreading technique,” *RSC Advances*, 13(15), p. 9933. Available at: https://doi.org/10.1039/D3RA00102D.

Li, T. *et al.* (2024) “Development of membrane-targeting TPP+-chloramphenicol conjugates to combat methicillin-resistant staphylococcus aureus (MRSA) infections,” *European Journal of Medicinal Chemistry*, 264, p. 115973. Available at: https://doi.org/10.1016/J.EJMECH.2023.115973.

Li, X. *et al.* (2008) *Application of gyrB in the identification of closely related bacteria--a review*. Available at: https://www.researchgate.net/publication/51425774_Application_of_gyrB_in_the_identification_of_closely_related_bacteria--_a_review (Accessed: October 5, 2025).

Lin, J. *et al.* (2022) “Removal of chloramphenicol antibiotics in natural and engineered water systems: Review of reaction mechanisms and product toxicity,” *Science of The Total Environment*, 850, p. 158059. Available at: https://doi.org/10.1016/J.SCITOTENV.2022.158059.

Liu, G. *et al.* (2018) “Advances in applications of rhamnolipids biosurfactant in environmental remediation: A review,” *Biotechnology and Bioengineering*, 115(4), pp. 796–814. Available at: https://doi.org/10.1002/bit.26517.

Liu, Q. *et al.* (2015) “Production of surfactin isoforms by Bacillus subtilis BS-37 and its applicability to enhanced oil recovery under laboratory conditions,” *Biochemical Engineering Journal*, 93, pp. 31–37. Available at: https://doi.org/10.1016/J.BEJ.2014.08.023.

Luketich, M., Samouei, H. and Nasrabadi, H. (2024) “A novel approach to produced water management using surfactants for water-wise energy production,” *Geoenergy Science and Engineering*, 242, p. 213187. Available at: https://doi.org/10.1016/J.GEOEN.2024.213187.

MCCURDY, P.R. and Gieschen, M.M. (1963) “Plasma Concentration of Chloramphenicol and Bone Marrow Suppression,” *Blood*, 21(3), pp. 363–372. Available at: https://doi.org/10.1182/BLOOD.V21.3.363.363.

Mohamad Shukri, I.A. *et al.* (2025) “Rhamnolipids as an antifungal agent against Rhizoctonia solani USM-PD2 causing sheath blight disease of paddy,” *Biocatalysis and Agricultural Biotechnology*, 64, p. 103511. Available at: https://doi.org/10.1016/J.BCAB.2025.103511.

Mukhia, S. *et al.* (2022) “Multilocus sequence based identification and adaptational strategies of *Pseudomonas* sp. from the supraglacial site of Sikkim Himalaya,” *PLoS ONE*, 17(1 1). Available at: https://doi.org/10.1371/JOURNAL.PONE.0261178

Mulligan, C.N. (2005) “Environmental applications for biosurfactants,” *Environmental Pollution*, 133(2), pp. 183–198. Available at: https://doi.org/10.1016/J.ENVPOL.2004.06.009.

Mulligan, C.N.., Mudhoo, Ackmez. and Sharma, S.K.. (2014) “Biosurfactants : research trends and applications.”

Onlamool, T., Saimmai, A. and Maneerat, S. (2022) “Antifungal Activity of Rhamnolipid Biosurfactant Produced by *Pseudomonas aeruginosa* A4 against Plant Pathogenic Fungi,” *Trends in Sciences*, 20(3), p. 6524. Available at: https://doi.org/10.48048/tis.2023.6524.

Pamp, S.J. and Tolker-Nielsen, T. (2007) “Multiple roles of biosurfactants in structural biofilm development by *Pseudomonas aeruginosa*,” *Journal of bacteriology*, 189(6), pp. 2531–2539. Available at: https://doi.org/10.1128/JB.01515-06.

Perinelli, D.R. *et al.* (2020) “Surfactant Self-Assembling and Critical Micelle Concentration: One Approach Fits All?,” *Langmuir*, 36(21), pp. 5745–5753. Available at: https://doi.org/10.1021/ACS.LANGMUIR.0C00420/SUPPL_FILE/LA0C00420_SI_001.PDF.

Poole, K. (2005) “Efflux-mediated antimicrobial resistance,” *The Journal of antimicrobial chemotherapy*, 56(1), pp. 20–51. Available at: https://doi.org/10.1093/JAC/DKI171.

Puangseree, J. *et al.* (2024) “Molecular basis of the persistence of chloramphenicol resistance among Escherichia coli and Salmonella spp. from pigs, pork and humans in Thailand,” *PloS one*, 19(5). Available at: https://doi.org/10.1371/JOURNAL.PONE.0304250.

Rahal, J.J. and Simberkoff, M.S. (1979) “Bactericidal and bacteriostatic action of chloramphenicol against meningeal pathogens,” *Antimicrobial Agents and Chemotherapy*, 16(1), pp. 13–18. Available at: https://doi.org/10.1128/AAC.16.1.13.

Ramesh, M. and Sakthishobana, K. (2021) “Significance of biosurfactants in oil recovery and bioremediation of crude oil,” *Green Sustainable Process for Chemical and Environmental Engineering and Science: Biosurfactants for the Bioremediation of Polluted Environments*, pp. 211–226. Available at: https://doi.org/10.1016/B978-0-12-822696-4.00006-1.

Rampioni, G. *et al.* (2006) “The quorum-sensing negative regulator RsaL of *Pseudomonas aeruginosa* binds to the lasI promoter,” *Journal of Bacteriology*, 188(2), pp. 815–819. Available at: https://doi.org/10.1128/JB.188.2.815-819.2006/ASSET/414D6EF5-B77D-402E-BEC1-EDB8887D423A/ASSETS/GRAPHIC/ZJB0020653830005.JPEG.

Rani, M., Weadge, J.T. and Jabaji, S. (2020) “Isolation and Characterization of Biosurfactant-Producing Bacteria From Oil Well Batteries With Antimicrobial Activities Against Food-Borne and Plant Pathogens,” *Frontiers in Microbiology*, 11, p. 64. Available at: https://doi.org/10.3389/FMICB.2020.00064/FULL.

Safari, P. *et al.* (2023) “Evaluation of surface activity of rhamnolipid biosurfactants produced from rice bran oil through dynamic surface tension,” *Journal of Petroleum Exploration and Production Technology*, 13(10), pp. 2139–2153. Available at: https://doi.org/10.1007/S13202-023-01660-Z/FIGURES/1.

Sanga, B. and Kharel, M.K. (2023) “Chloramphenicol,” *Encyclopedia of Toxicology, Fourth Edition: Volume 1-9*, 2, pp. V2-825-V2-830. Available at: https://doi.org/10.1016/B978-0-12-824315-2.00349-3.

Dos Santos, R. (2020) *BIOPROSPECÇÃO DE BACTÉRIAS DEGRADADORAS DE PETRÓLEO*. Universidad Estadual Do Norte Fluminense Darcy Ribeiro. Available at: https://uenf.br/posgraduacao/producao-vegetal/wp-content/uploads/sites/10/2021/06/TeseFinal-Renata-Soares-dos-Santos-1.pdf (Accessed: August 26, 2024).

Sarker, S.D., Nahar, L. and Kumarasamy, Y. (2007) “Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals,” *Methods*, 42(4), pp. 321–324. Available at: https://doi.org/10.1016/J.YMETH.2007.01.006.

Sarmah, A.K., Meyer, M.T. and Boxall, A.B.A. (2006) “A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment,” *Chemosphere*, 65(5), pp. 725–759. Available at: https://doi.org/10.1016/j.chemosphere.2006.03.026.

Sarubbo, L.A. *et al.* (2022) “Biosurfactants: Production, properties, applications, trends, and general perspectives,” *Biochemical Engineering Journal*, 181, p. 108377. Available at: https://doi.org/10.1016/J.BEJ.2022.108377.

Schmidberger, A. *et al.* (2013) “Expression of genes involved in rhamnolipid synthesis in *Pseudomonas aeruginosa* PAO1 in a bioreactor cultivation,” *Applied Microbiology and Biotechnology*, 97(13), pp. 5779–5791. Available at: https://doi.org/10.1007/S00253-013-4891-0/FIGURES/9.

Sen, S. *et al.* (2020) “Rhamnolipid exhibits anti-biofilm activity against the dermatophytic fungi Trichophyton rubrum and Trichophyton mentagrophytes,” *Biotechnology Reports*, 27, p. e00516. Available at: https://doi.org/10.1016/J.BTRE.2020.E00516.

Sonmez, V.Z., Akarsu, C. and Sivri, N. (2025) “Rhamnolipid: nature-based solution for the removal of microplastics from the aquatic environment,” *Integrated environmental assessment and management*, 21(2), pp. 350–359. Available at: https://doi.org/10.1093/INTEAM/VJAE037.

Sousa, A.M., Pereira, M.J. and Matos, H.A. (2022) “Oil-in-water and water-in-oil emulsions formation and demulsification,” *Journal of Petroleum Science and Engineering*, 210, p. 110041. Available at: https://doi.org/10.1016/J.PETROL.2021.110041.

Tan, Y.N. and Li, Q. (2018) “Microbial production of rhamnolipids using sugars as carbon sources,” *Microbial Cell Factories*, 17(1), p. 89. Available at: https://doi.org/10.1186/S12934-018-0938-3.

Thakur, P. *et al.* (2021) “Rhamnolipid the Glycolipid Biosurfactant: Emerging trends and promising strategies in the field of biotechnology and biomedicine,” *Microbial Cell Factories*, 20(1), pp. 1–15. Available at: https://doi.org/10.1186/S12934-020-01497-9/FIGURES/12.

Touabi, L. *et al.* (2025) “Antiviral Activity of Rhamnolipids Nano-Micelles Against Rhinoviruses—In Silico Docking, Molecular Dynamic Analysis and In-Vitro Studies,” *Current Issues in Molecular Biology*, 47(5), p. 333. Available at: https://doi.org/10.3390/CIMB47050333/S1.

Vittal, R., Gomathi, H. and Kim, K.J. (2006) “Beneficial role of surfactants in electrochemistry and in the modification of electrodes,” *Advances in Colloid and Interface Science*, 119(1), pp. 55–68. Available at: https://doi.org/10.1016/J.CIS.2005.09.004.

Weeks, J.L., Mason, E.O. and Baker, C.J. (1981) “Antagonism of ampicillin and chloramphenicol for meningeal isolates of group B streptococci,” *Antimicrobial Agents and Chemotherapy*, 20(3), pp. 281–285. Available at: https://doi.org/10.1128/AAC.20.3.281.

Wen, X. *et al.* (2016) “Limitations of MIC as sole metric of pharmacodynamic response across the range of antimicrobial susceptibilities within a single bacterial species,” *Scientific Reports*, 6(1), pp. 1–8. Available at: https://doi.org/10.1038/SREP37907;SUBJMETA.

Wiegand, I., Hilpert, K. and Hancock, R.E.W. (2008) “Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances,” *Nature protocols*, 3(2), pp. 163–175. Available at: https://doi.org/10.1038/NPROT.2007.521.

White, R.L. et al. (1996) Comparison of Three Different In Vitro Methods of Detecting Synergy: Time-Kill, Checkerboard, and E test, ANTIMICROBIAL AGENTS AND CHEMOTHERAPY. Available at: https://journals.asm.org/journal/aac.

Wu, B. *et al.* (2022) “Research advances of microbial enhanced oil recovery,” *Heliyon*, 8(11), p. e11424. Available at: https://doi.org/10.1016/J.HELIYON.2022.E11424.

Yamamoto, S. and Harayama, S. (1995) “PCR amplification and direct sequencing of gyrB genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas* putida strains,” *Applied and Environmental Microbiology*, 61(3), pp. 1104–1109. Available at: https://doi.org/10.1128/AEM.61.3.1104-1109.1995.

Yang, X. *et al.* (2020) “Sub-CMC solubilization of n-alkanes by rhamnolipid biosurfactant: the Influence of rhamnolipid molecular structure,” *Colloids and Surfaces B: Biointerfaces*, 192, p. 111049. Available at: https://doi.org/10.1016/J.COLSURFB.2020.111049.

Youssef, N.H. *et al.* (2004) “Comparison of methods to detect biosurfactant production by diverse microorganisms,” *Journal of Microbiological Methods*, 56(3), pp. 339–347. Available at: https://doi.org/10.1016/J.MIMET.2003.11.001

.

Zhang, G.L. *et al.* (2005) “Biodegradation of crude oil by *Pseudomonas aeruginosa* in the presence of rhamnolipids,” *Journal of Zhejiang University. Science. B*, 6(8), p. 725. Available at: https://doi.org/10.1631/JZUS.2005.B0725.

Zhu, P. *et al.* (2022) “Rhamnolipids from non-pathogenic Acinetobacter calcoaceticus: Bioreactor-scale production, characterization and wound healing potency,” *New Biotechnology*, 67, pp. 23–31. Available at: https://doi.org/10.1016/J.NBT.2021.12.001.

Zia, S. *et al.* (2023) “Resistance Modulation of Individual and Polymicrobial Culture of S. aureus and E. coli through Nanoparticle-Coupled Antibiotics,” *Biomedicines*, 11(11). Available at: https://doi.org/10.3390/BIOMEDICINES11112988.