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EXPLORING THE POTENTIAL OF NATIVE MICROORGANISMS FROM DIESEL-CONTAMINATED AREAS FOR ENHANCED PETROLEUM HYDROCARBON DEGRADATION

Florianópolis, 2025

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RESUMO

A introdução do biodiesel na matriz energética brasileira representa um avanço significativo em direção a fontes de energia mais sustentáveis. Por outro lado, o aumento do uso de composições binárias, como o diesel B20 (20% de biodiesel e 80% de diesel v/v), traz consigo o risco de contaminações ambientais resultante de eventuais derramamentos. Apesar do biodiesel ser suscetível à degradação microbiana, o óleo diesel mineral que compõe a maior parcela das misturas binárias, é composto por um conjunto complexo de hidrocarbonetos alifáticos e aromáticos, reconhecidos pela sua toxicidade e persistência ambiental. Nesse contexto, a biorremediação, por meio do uso estratégico de microrganismos na recuperação de áreas impactadas por petroquímicos, apresenta-se como uma alternativa de baixo custo que permite a remediação permanente de contaminantes residuais. Sob essa perspectiva, este estudo objetivou coletar amostras em áreas experimentais contaminadas por diesel B20, prospectar espécies microbianas nativas e formular consórcios microbianos compatíveis com características metabólicas associadas à degradação aprimorada de hidrocarbonetos, incluindo a análise da produção de biossurfactantes e enzimas como a lacase. A análise cromatográfica (CG) das amostras de solo da área P05 do REMA revelou que, mesmo uma década após a liberação desses contaminantes, concentrações residuais de diversos hidrocarbonetos policíclicos aromáticos (HPAs) e níveis elevados de hidrocarbonetos do grupo BTEX, como o benzeno, tolueno e xilenos, foram encontradas. Para área P10, valores acima da média recomendada foram verificados para os hidrocarbonetos xilenos, tolueno, naftaleno e antraceno. Um total de 19 isolados, representados por 7 fungos e 12 bactérias, foram obtidos após o enriquecimento das amostras de solo em meio mínimo, contendo óleo diesel como única fonte de carbono. A análise de caracteres macro- e micromorfológicos, em conjunto com o sequenciamento de regiões parciais dos genes 16S rRNA, Calmodulina *(CaM)* e β-tubulina *(BenA)*, permitiu a identificação dos isolados. O teste do colapso da gota, demonstrou que todos os fungos produzem moléculas tensoativas, com destaque para a linhagem de *Trichoderma koningiopsis* P05R2, que reduziu a tensão superficial do meio para 37,4 mN/m. A análise quantitativa da produção de lacases, evidenciou um rápido crescimento seguido por percentuais elevados de atividade enzimática para *Stenotrophomonas maltophilia* P05R11 (0,917 U/mL) e *Trichoderma koningiopsis* P05R2 (0,425 U/mL). Após os testes de antagonismo, quatro consórcios compatíveis foram formulados. A análise da degradação de HPAs e HTPs (C5-C40) presentes no óleo diesel revelou que os consórcios apresentaram uma capacidade de degradação significativamente superior em comparação com as cepas individuais. Os melhores resultados foram observados para um consórcio misto bacteriano-fúngico, composto por *Trichoderma koningiopsis* P05R2, *Serratia marcescens* P10R19 e *Burkholderia cepacia* P05R9, que apresentou um espectro de degradação ≥91% para todos os onze HPAs analisados, removendo 93,61% dos HPAs totais e 93,52% dos HTP (C5-C40)**.** Esse achado destaca o potencial sinérgico entre bactérias e fungos como uma abordagem eficaz para a remoção de hidrocarbonetos complexos. Além disso, este estudo apresenta o primeiro relato de *Trichoderma koningiopsis* como um candidato à biorremediação, fornecendo bases científicas para o aprimoramento de tecnologias sustentáveis voltadas à recuperação de ambientes contaminados.

Palavras-chave: Consórcios bacterianos; biodegradação; co-culturas; diesel B20; consórcios fúngicos; hidrocarbonetos de petróleo; HPA.

ABSTRACT

The introduction of biodiesel into the Brazilian energy matrix represents a significant step toward more sustainable energy sources. However, the increasing use of binary fuel blends, such as B20 diesel (20% biodiesel and 80% diesel v/v), raises concerns regarding environmental contamination resulting from potential spills. Although biodiesel is susceptible to microbial degradation, mineral diesel, which constitutes the majority of these blends, is composed of a complex mixture of aliphatic and aromatic hydrocarbons known for their toxicity and environmental persistence. In this context, bioremediation, through the strategic use of microorganisms in the recovery of areas impacted by petrochemical contaminants, emerges as a cost-effective alternative that enables the permanent removal of residual pollutants. Under this perspective, this study aimed to collect samples from experimental sites contaminated with B20 diesel, prospect native microbial species, and formulate microbial consortia compatible with metabolic traits associated with enhanced hydrocarbon degradation, including the analysis of biosurfactant and laccase enzyme production. Chromatographic analysis (GC) of soil samples from the P05 area of REMA revealed that, even a decade after the release of these contaminants, residual concentrations of various polycyclic aromatic hydrocarbons (PAHs) and elevated levels of BTEX-group hydrocarbons, such as benzene, toluene, and xylenes, were detected. In the P10 area, levels exceeding the recommended average were observed for xylenes, toluene, naphthalene, and anthracene. A total of 19 microbial isolates, comprising 7 fungi and 12 bacteria, were obtained following the enrichment of soil samples in a minimal medium with diesel oil as the sole carbon source. Macro- and micromorphological characterization, combined with the sequencing of partial regions of the 16S rRNA, Calmodulin (*CaM*)**,** and β-tubulin (*BenA*) genes, enabled the identification of these isolates. The drop-collapse assay demonstrated that all fungal strains produced surfactant molecules, with *Trichoderma koningiopsis* P05R2 standing out by reducing the medium's surface tension to 37.4 mN/m. Quantitative analysis of laccase production revealed rapid growth followed by high enzymatic activity levels, particularly in *Stenotrophomonas maltophilia* P05R11 (0.917 U/mL) and *Trichoderma koningiopsis* P05R2 (0.425 U/mL). Following antagonistic interaction tests, four compatible microbial consortia were formulated. The degradation analysis of PAHs and total petroleum hydrocarbons (TPHs, C5-C40) present in diesel oil demonstrated that the consortia exhibited significantly higher degradation capacities compared to individual strains. The most promising results were observed for a mixed bacterial-fungal consortium composed of *Trichoderma koningiopsis* P05R2, *Serratia marcescens* P10R19, and *Burkholderia cepacia* P05R9, which achieved a degradation spectrum of ≥91% for all eleven analyzed PAHs, removing 93.61% of total PAHs and 93.52% of TPH (C5-C40)**.** This finding highlights the synergistic potential between bacteria and fungi as an effective strategy for the removal of complex hydrocarbons. Furthermore, this study presents the first report of *Trichoderma koningiopsis* as a candidate for bioremediation, providing a scientific basis for the advancement of sustainable technologies aimed at the recovery of contaminated environments.

Keywords: Bacterial consortia; biodegradation; co-cultures; diesel B20; fungal consortia; petroleum hydrocarbons; PAHs.

RESUMO EXPANDIDO

Introdução

A contaminação ambiental por hidrocarbonetos de petróleo é um dos principais desafios ecológicos da atualidade, devido à sua ampla utilização industrial, complexidade química e persistência no meio ambiente. Entre esses compostos, os hidrocarbonetos policíclicos aromáticos (HPAs) destacam-se pela sua elevada toxicidade, potencial mutagênico e carcinogênico, além de sua resistência à degradação natural. Métodos físico-químicos de remediação, embora amplamente utilizados, apresentam limitações, como altos custos e impactos ambientais adversos.

Nesse contexto, a introdução do biodiesel na matriz energética brasileira representa um avanço significativo em direção a fontes de energia mais sustentáveis. No entanto, o aumento do uso de composições binárias, como o diesel B20 (20% de biodiesel e 80% de diesel v/v), eleva o risco de contaminações ambientais resultantes de eventuais derramamentos. Embora o biodiesel seja suscetível à degradação microbiana, o óleo diesel mineral, que compõe a maior parte dessas misturas, contém uma complexa matriz de hidrocarbonetos alifáticos e aromáticos, reconhecidos por sua toxicidade e persistência ambiental. Assim, mesmo com a adoção de combustíveis renováveis, a presença desses poluentes no meio ambiente continua sendo um problema relevante, demandando estratégias eficazes para sua mitigação.

Diante dessa problemática, a biorremediação microbiana surge como uma alternativa sustentável e economicamente viável, aproveitando a capacidade de determinados microrganismos de degradar hidrocarbonetos e convertê-los em subprodutos menos tóxicos. O uso de consórcios microbianos tem se mostrado uma abordagem promissora para otimizar a degradação desses poluentes, combinando a ação metabólica de diferentes espécies em processos sinérgicos. Enquanto algumas bactérias e fungos produzem biossurfactantes, que aumentam a biodisponibilidade dos hidrocarbonetos, outras sintetizam enzimas oxidativas, como lacases e peroxidases, que catalisam a degradação desses compostos. Dessa forma, a formulação estratégica de consórcios microbianos pode aumentar significativamente a eficiência da biorremediação de áreas contaminadas.

Com base nessa perspectiva, a presente pesquisa teve como objetivo geral a prospecção de espécies microbianas a partir de amostras de solo de áreas experimentais contaminadas por misturas de diesel B20**,** visando o desenvolvimento de consórcios e avaliando seu potencial na degradação de HPAs e hidrocarbonetos totais de petróleo (HTP).

Objetivos

O estudo teve como principal objetivo o desenvolvimento de consórcios microbianos destinados à biorremediação de hidrocarbonetos de petróleo. Os objetivos específicos incluem: (1) Coletar solos contaminados com óleo diesel B20 para isolamento e prospecção de linhagens microbianas degradadoras de hidrocarbonetos por meio de ensaios de enriquecimento; (2) Caracterizar os isolados microbianos com base em aspectos macro e micromorfológicos; (3) Avaliar a diversidade filogenética entre os isolados bacterianos por meio da amplificação de sequências repetitivas BOX-PCR; (4) Identificar espécies bacterianas e fúngicas através do sequenciamento de regiões parciais do gene 16S RNAr, β-Tubulina (*BenA*) e Calmodulina (*CaM*); (5) Selecionar e avaliar microrganismos com potencial para produção de biossurfactantes e lacases; (6) Formular consórcios microbianos com base na compatibilidade entre espécies, utilizando ensaios de interação antagônica; (7) Testar a eficiência dos consórcios

formulados na degradação de HPAs e HTP em experimentos de microcosmo líquido contendo óleo diesel como fonte de hidrocarbonetos.

Metodologia

O estudo foi estruturado em três capítulos, cada um abordando aspectos distintos, porém interconectados, da degradação microbiana de hidrocarbonetos. O **Capítulo 1** apresenta uma revisão abrangente da literatura sobre a biodegradação microbiana de hidrocarbonetos e as espécies envolvidas, com ênfase particular na degradação de HPAs e o papel de linhagens bacterianas e fúngicas no metabolismo desses compostos. Este capítulo explora os principais mecanismos enzimáticos envolvidos nesse processo, bem como a emulsificação de hidrocarbonetos mediada por biossurfactantes, que, em conjunto, contribuem para a eficiência da biorremediação. Propondo estratégias de triagem para o isolamento e seleção eficiente de microrganismos degradadores de hidrocarbonetos, a partir de diferentes metodologias para a identificação de linhagens com alto potencial metabólico. Além disso, discute a formulação de consórcios microbianos como uma abordagem estratégica para otimizar a degradação de hidrocarbonetos, destacando os benefícios da sinergia microbiana e das vias metabólicas complementares. O **Capítulo 2** apresenta o estudo experimental realizado para desenvolver e avaliar consórcios microbianos destinados à biorremediação de hidrocarbonetos de petróleo. Neste estudo, cepas bacterianas e fúngicas foram isoladas a partir de locais experimentais intencionalmente contaminados com biodiesel B20 (20% biodiesel, 80% diesel), simulando cenários reais de contaminação. Um *screening framework* sistemático foi aplicado para avaliar o potencial desses isolados na degradação de hidrocarbonetos, com foco em características funcionais-chave, como atividade extracelular de lacases, produção de biossurfactantes e compatibilidade entre espécies. Após essa triagem inicial, as cepas selecionadas foram estrategicamente combinadas na formulação de consórcios microbianos e, posteriormente, testadas quanto à sua eficiência na degradação do óleo diesel, com especial atenção para hidrocarbonetos policíclicos aromáticos (HPAs) e hidrocarbonetos totais de petróleo (HTP). Enquanto o **Capítulo 3** amplia o escopo das descobertas apresentadas nos capítulos anteriores, transcendendo a análise experimental para refletir sobre as implicações mais profundas do uso de consórcios microbianos na biorremediação de hidrocarbonetos. Este capítulo não apenas conclui a jornada científica empreendida ao longo do estudo, mas também a amplia, lançando questionamentos fundamentais sobre os rumos da biotecnologia ambiental.

Resultados e Discussão

A revisão bibliográfica realizada no Capítulo 1 reforçou a importância das interações microbianas na degradação de hidrocarbonetos e destacou o potencial da formulação de consórcios para melhorar a eficiência da biorremediação. A literatura apontou que consórcios bacteriano-fúngicos podem degradar compostos de alta recalcitrância mais eficazmente do que microrganismos isolados, aproveitando vias metabólicas complementares. Além disso, ao propor uma estratégia de triagem para a prospecção de linhagens microbianas hidrocarbonoclásticas, esta revisão busca contribuir para o desenvolvimento de soluções inovadoras e sustentáveis para a biorremediação de ambientes contaminados por HPAs. No Capítulo 2, o estudo experimental confirmou a relevância dos achados da revisão bibliográfica, demonstrando que consórcios específicos apresentam taxas de degradação significativamente superiores em comparação aos isolados individuais. A análise por cromatografia gasosa (CG) das amostras de solo da área P05 do REMA revelou que, mesmo uma década após a liberação desses contaminantes, concentrações residuais de diversos hidrocarbonetos policíclicos

aromáticos (HPAs) e níveis elevados de hidrocarbonetos do grupo BTEX, como o benzeno, tolueno e xilenos, foram encontradas. Para área P10, valores acima da média recomendada foram verificados para os hidrocarbonetos xilenos, tolueno, naftaleno e antraceno. Um total de 19 isolados microbianos, sendo 7 fungos e 12 bactérias, foram obtidos após o enriquecimento das amostras de solo em meio mínimo contendo óleo diesel como única fonte de carbono. A caracterização macro- e micromorfológica, aliada ao sequenciamento de regiões parciais dos genes 16S rRNA**,** Calmodulina (*CaM*) e β-tubulina (*BenA*), possibilitou a identificação das espécies isoladas. Entre os fungos identificados, destacam-se *Penicillium simplicissimum* (n=1), *Trichoderma koningiopsis* (n=1), *Penicillium Janthinellum* (n=2) e *Penicillium pulvillorum* (n=3). Já entre as bactérias, foram identificadas *Bacillus cereus* (n=2), *Burkholderia cepacia* (n=3), *Bacillus* sp. (n=1), *Stenotrophomonas maltophilia* (n=1), *Dyella japonica* (n=1), *Paraburkholderia* sp. (n=1), *Burkholderia* sp. (n=2) e *Serratia marcescens* (n=1). Os ensaios funcionais demonstraram um elevado potencial biotecnológico dos isolados. O teste de colapso da gota revelou que todos os fungos produzem moléculas tensoativas, sendo que *Trichoderma koningiopsis* P05R2 apresentou o maior desempenho, reduzindo a tensão superficial do meio para 37,4 mN/m. Além disso, a análise quantitativa da produção de lacases evidenciou altos níveis de atividade enzimática para *Stenotrophomonas maltophilia* P05R11 (0,917 U/mL) e *Trichoderma koningiopsis* P05R2 (0,425 U/mL), reforçando o papel dessas espécies na degradação oxidativa de hidrocarbonetos. Com base nesses resultados, consórcios microbianos foram formulados estrategicamente, combinando espécies com alta produção de biossurfactantes e enzimas oxidativas para otimizar a degradação de hidrocarbonetos. Entre os consórcios testados, a associação de *Trichoderma koningiopsis* P05R2*, Serratia marcescens* P10R19 *e Burkholderia cepacia* P05R9 (consórcio misto FBB1) obteve a melhor performance, alcançando uma eficiência de degradação superior a 91% para todos os 11 HPAs analisados. Esse achado evidencia a eficácia das interações microbianas na biotransformação desses compostos, destacando o potencial sinérgico entre bactérias e fungos na biorremediação de hidrocarbonetos. Além disso, a identificação de *Trichoderma koningiopsis* como um candidato inédito para biorremediação amplia as perspectivas de aplicação desse fungo em processos de descontaminação ambiental, principalmente devido à sua capacidade de produção simultânea de biossurfactantes, lacases e sinergismo com as demais espécies do consórcio. O Capítulo 3 nos convida a olhar além dos experimentos e enxergar a biotecnologia como um reflexo da própria dinâmica da vida. A natureza, com seus sistemas interconectados, ensina que resiliência e adaptação surgem da cooperação, um princípio exemplificado pelos consórcios microbianos testados. A descoberta de *Trichoderma koningiopsis* como um candidato promissor para biorremediação reforça a importância da bioprospecção contínua e do respeito à biodiversidade. Mais do que uma solução para contaminações específicas, este trabalho sugere um modelo de inovação que se espelha na natureza, onde o avanço científico não é uma mera intervenção, mas uma extensão dos mecanismos que já regem os ecossistemas. Dessa forma, a biotecnologia ambiental se torna não apenas uma ferramenta de mitigação, mas um caminho para reequilibrar a relação entre humanidade e meio ambiente.

Considerações Finais

Além de fornecer um guia estruturado para a triagem e seleção de microrganismos degradadores, os resultados obtidos no estudo prático reforçam a premissa de que consórcios microbianos desempenham um papel essencial na biorremediação, ampliando o espectro e a eficiência da degradação de compostos complexos. A capacidade dessas interações microbianas de potencializar a transformação e mineralização de hidrocarbonetos destaca a importância da formulação estratégica de consórcios, fornecendo bases científicas para o aprimoramento de tecnologias sustentáveis voltadas à recuperação de ambientes contaminados.

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PAH – Polycyclic Aromatic Hydrocarbon

TPH – Total Petroleum Hydrocarbon

Alk - Alkane Hydroxylases

TCA - Tricarboxylic Acid Cycle

CYP - Cytochrome P450 Monooxygenases

Bap - Benzo[A]Pyrene

BH - Bushnell-Haas

MSM - Minimal Salt Medium

DCPIP - 2,6-Dichlorophenolindophenol

MATH - Microbial Adhesion To Hydrocarbons

E.I - Emulsification Index

E24 - Emulsification Index

LaC – Laccase

MnP – Manganese Peroxidases

LiP - Lignin Peroxidases

VP - Versatile Peroxidases

MEA – Malt Extract Agar

RBBR – Remazol Brilliant Blue R

ABTS - 2,2′-Azino-Bis(3-Ethylbenzothiazoline-6-Sulfonic Acid

DMP - 2,6-Dimethoxyphenol

LMW – Low-Molecular-Weight

HMW - High-Molecular-Weight

PVA - Polyvinyl Alcohol

FID - Flame Ionization Detector

BTEX - Benzene, Toluene, Ethylbenzene and Xylenes

NA - Nutrient Agar

CYA - Czapek Yeast Extract Agar

YES - Yeast Extract Sucrose Agar

ML - Maximum Likelihood

NJ - Neighbor-Joining

STR - Surface Tension Reduction

O.D - Optical Density

CONAMA - National Environment Council

SUMÁRIO

1. INTRODUCTION

Petroleum hydrocarbons represent a persistent environmental challenge due to their widespread industrial use, chemical complexity, and resistance to natural degradation (Bora; Anand; Tech, 2018; Elijah, 2022). As fundamental components of the global energy matrix, petroleum and its derivatives continue to be essential for economic and industrial activities, accounting for a significant portion of the world's primary energy consumption (IEA, 2021; Al-Rubaye et al., 2023). However, the extraction, transportation, refining, and utilization of petroleum products have resulted in frequent contamination events, primarily through accidental spills and chronic discharges (Akhmiyeva, 2021; Hazaimeh; Ahmed, 2021). Among the hydrocarbon fractions, polycyclic aromatic hydrocarbons (PAHs) are of particular concern due to their toxicity, carcinogenic and mutagenic potential, and environmental recalcitrance (Mastrangelo; Fadda; Marzia, 1996; Varjani et al., 2018; Wassenaar; Verbruggen, 2021).

Given the environmental persistence and hazardous nature of these compounds, effective remediation strategies are essential for mitigating their impact (Kuppusamy et al., 2020; Ossai et al., 2020). Traditional physicochemical approaches often involve high costs, low efficiency in removing residual contamination, and potential secondary pollution (Chukwunonso Ossai; Shahul Hamid; Hassan, 2022; Gaur; Gupta; Pandey, 2022). In this context, microbial bioremediation has emerged as a promising alternative, offering a cost-effective and environmentally sustainable approach to hydrocarbon degradation (Ahmed; Fakhruddin, 2018; Mahmoud; Bagy, 2019). Certain bacterial and fungal species possess metabolic pathways that enable them to utilize hydrocarbons as a carbon source, transforming these pollutants into less harmful end products (Seo; Keum; Li, 2009; Li; Liu; Gadd, 2020; Mangla et al., 2021).

However, while individual strains have demonstrated hydrocarbon degradation capabilities, microbial consortia, comprising synergistic interactions between bacterial, fungal, or mixed fungal-bacterial species, exhibit superior efficiency in degrading complex hydrocarbon mixtures such as PAHs (Li; Li, 2011; Unimke; Mmuoegbulam; Anika, 2018; Silva Monteiro et al., 2024). These consortia leverage enzymatic complementarity, biosurfactant production, and metabolic cooperation to enhance bioremediation efficiency (Zeng et al., 2011; Martinho et al., 2019; Annuar et al., 2023). Despite their potential, the effective selection and formulation of microbial consortia require systematic methodologies that integrate strain compatibility, enzymatic profiling, and metabolic interactions.

To address these challenges, this thesis is structured into three main chapters, each addressing distinct but interconnected aspects of microbial hydrocarbon degradation: **Chapter 1** presents a comprehensive literature review on microbial species and mechanisms involved in hydrocarbon biodegradation, with a particular focus on PAHs and the pivotal role of bacterial and fungal strains in the metabolic pathways responsible for their degradation. This chapter explores key enzymatic mechanisms, as well as biosurfactant-mediated hydrocarbon emulsification, which collectively enhance bioremediation efficiency. Furthermore, it presents a structured *screening framework* for the efficient isolation and selection of hydrocarbon-degrading microorganisms, integrating advanced methodologies to identify strains with high metabolic potential. Additionally, it discusses the formulation of microbial consortia as a strategic approach to optimizing hydrocarbon degradation, highlighting the benefits of microbial synergy and complementary metabolic pathways. **Chapter 2** presents the experimental study conducted to develop and evaluate microbial consortia for the bioremediation of petroleum hydrocarbons. In this study, bacterial and fungal strains were isolated from experimental sites intentionally contaminated with B20 biodiesel (20% biodiesel, 80% diesel), simulating real-world contamination scenarios. A systematic *screening framework* was applied to assess the potential of these isolates for hydrocarbon degradation, focusing on key functional traits such as extracellular laccase activity, biosurfactant production, and species compatibility. Following this initial screening, selected strains were strategically assembled into microbial consortia and subsequently tested for their efficiency in degrading diesel oil, with particular attention to PAHs and total petroleum hydrocarbons (TPHs). While **Chapter 3** expands the scope of the findings presented in the previous chapters, it transcends experimental analysis to reflect on the broader implications of microbial consortia in hydrocarbon bioremediation. This chapter not only synthesizes the scientific journey undertaken throughout the study but also extends it, posing fundamental questions about the future directions of environmental biotechnology.

Following these insights, the present study tested the following hypotheses: first, microbial consortia composed of bacterial, fungal, or mixed bacterial-fungal species exhibit higher degradation efficiency of PAHs and TPHs compared to individual strains, leveraging synergistic metabolic interactions; second, the selection and strategic assembly of microbial consortia based on the production of biosurfactants and oxidative enzymes (e.g., laccases) enhance hydrocarbon degradation efficiency, broaden the degradation spectrum.

By integrating literature-based insights with experimental findings, this thesis aims to contribute to the advancement of microbial bioremediation strategies, emphasizing the importance of microbial consortia in addressing hydrocarbon contamination. The findings provide a basis for future research and potential applications in environmental restoration, supporting the transition toward more sustainable approaches for mitigating petroleum-derived pollution.

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2. OBJECTIVES

2.1 GENERAL OBJECTIVE

This project aims to develop microbial consortia by prospecting native species from environments impacted by petroleum hydrocarbons, with the ultimate goal of utilizing them in bioremediation processes.

2.2 SPECIFIC OBJECTIVES

I. Review and systematically catalog hydrocarbon-degrading microorganisms, elucidating their metabolic mechanisms and the synergistic interactions that enhance the biotransformation of PAHs, with a focus on optimizing bioremediation strategies.

II. Review and develop a structured screening framework for the efficient selection of microbial strains with high bioremediation potential, integrating advanced methodologies for strain characterization, compatibility assessments, and microbial consortium optimization to maximize hydrocarbon degradation efficiency.

III. Collect contaminated soils with B20 diesel blend, to isolate and prospect microbial strains with hydrocarbon degradation potential through enrichment assays.

IV. Characterize the microbial isolates based on macro- and micromorphological traits.

V. Assess phylogenetic groupings among bacterial isolates through BOX-PCR amplification of repetitive sequences.

VI. Identify bacterial and fungal species through sequencing of partial regions of the 16S rRNA gene, β-Tubulin (*BenA*), and Calmodulin (*CaM*).

VII. Evaluate the microbial isolates for biosurfactant and laccase production.

VIII. Select compatible species for microbial consortium development through antagonistic interaction assays.

IX. Assess the degradation potential of polycyclic aromatic hydrocarbons (PAHs) by the formulated microbial consortia in liquid-phase microcosm experiments.

3. CHAPTER I

FROM SCREENING TO BIODEGRADATION: MICROBIAL STRATEGIES FOR PETROLEUM HYDROCARBON REMOVAL

This chapter corresponds to the submitted article: SILVA MONTEIRO, J. P.; DELGADO DUARTE, R. T.; JOSÉ GIACHINI, From Screening to Biodegradation: Microbial Strategies for Petroleum Hydrocarbon Removal. Sent to the Journal of Microbiology and Biotechnology.

ABSTRACT

Petroleum-derived hydrocarbons represent a significant environmental challenge due to their persistence, toxicity, and recalcitrance. Microbial bioremediation emerges as a sustainable and efficient strategy for mitigating contamination, leveraging the metabolic versatility of bacteria and fungi. This review provides a comprehensive analysis of key microbial species involved in hydrocarbon degradation, with a particular emphasis on polycyclic aromatic hydrocarbons (PAHs), recognized for their structural complexity and harmful effects. Key enzymatic pathways involved in the enhanced degradation of these compounds, including those mediated by laccases and peroxidases, are thoroughly explored, along with the role of biosurfactant production in improving hydrocarbon bioavailability. Special emphasis is placed on the formulation of microbial consortia, highlighting the synergistic interactions between bacterial and fungal species that expand the degradation spectrum and improve biodegradation efficiency. Given the advantages conferred by microbial consortia, this review introduces a systematic screening framework aimed at refining the selection of hydrocarbon-degrading microorganisms and optimizing microbial consortia formulation. Through the integration of strategic selection methodologies, this study contributes to the advancement of sustainable and efficient bioremediation technologies, reinforcing the critical role of microbial interactions in environmental restoration.

Keywords: *Bioremediation, polycyclic aromatic hydrocarbons (PAHs), bacterial consortia, fungal consortia, mixed consortia*

3.1. INTRODUCTION

As one of the most crucial raw materials for a wide range of industrial and commercial activities, petroleum and its byproducts continue to play a vital role in the global economy and energy matrix, accounting for approximately 30.95% of the global primary energy consumption (Cousar, 2014; Bp, 2022). The extensive set of anthropogenic activities related to oil extraction, transportation, refining, and consumption of its byproducts is directly associated with the widespread dissemination of hydrocarbons (Rama et al., 2019; Liu et al., 2022). Petroleum consists of a mixture of aliphatic and alicyclic hydrocarbons (linear, branched, saturated, and unsaturated), mono- or polyaromatic hydrocarbons, asphaltenes, and resins, along with small amounts of nitrogen, sulfur, and oxygen (Schobert, 2013). A specific group of 16 polycyclic aromatic hydrocarbons (PAHs), including benzo[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene, benzo[g,h,i]perylene, benzo[k]fluoranthene, chrysene, dibenz[a,h]anthracene, fluoranthene, fluorene, indeno[1,2,3-cd]pyrene, naphthalene, phenanthrene, and pyrene, has been listed by the United States Environmental Protection Agency as priority pollutants for study, due to their persistence in the environment resulting from the stability of their chemical structures against degradation. Generally, hydrocarbon

contamination negatively impacts human health and reduces the biotic potential of various ecosystems due to the toxicity, low solubility, recalcitrance, and carcinogenic and mutagenic potential of these compounds, making them the subject of numerous studies and environmental monitoring efforts (Vasudevan; Gayathri; Krishnan, 2018; Wen et al., 2021; Vijayanand et al., 2023).

Microbial bioremediation involves the use of microorganisms to remove, degrade, and/or transform pollutants into harmless substances such as carbon dioxide, water, inorganic salts (aerobic degradation), and methane (anaerobic degradation) (Dzionek; Wojcieszyńska; Guzik, 2016; Yadav et al., 2020). This technique is promising and offers lower costs compared to conventional methods, which can be inefficient for remediating areas contaminated with PAHs or require complex pre-treatments (Mrozik; Piotrowska-Seget, 2010; Gan et al., 2022). Microbial communities in both aquatic and terrestrial environments have shown adaptive responses to hydrocarbon exposures (Ławniczak et al., 2020). Although the initial impact often results in a decline of local populations, the stress induced by these contaminants selects for bacteria and fungi capable of utilizing hydrocarbons for their growth and reproduction (Bovio et al., 2017; Varjani; Gnansounou; Pandey, 2017; Imam et al., 2019), due to the secretion of oxidative and hydrolytic enzymes, and/or biosurfactants (Ferreira; Varjani; Taherzadeh, 2020; Zhang et al., 2020; Da Silva et al., 2023). Thus, the bioprospecting of strains isolated from contaminated environments has enabled the production of various useful metabolites (such as enzymes, biosurfactants, pigments, acids) or the use of the strains themselves as inoculants for environmental decontamination (Fulekar, 2017).

In sum, the success of microbial bioremediation depends on the use of robust inocula that possess a suitable metabolic system capable of consuming and mineralizing contaminants (Azubuike; Chikere; Okpokwasili, 2016; Pandolfo; Barra Caracciolo; Rolando, 2023). In this context, the use of microbial consortia allows for a broader exploitation of the individual performance of each strain during the degradation of complex hydrocarbons (Massot et al., 2022). This is because a single microorganism typically has the capacity to metabolize only a limited range of hydrocarbons. However, when acting in syntrophy, the combination of bacterial or fungal species, or their co-cultivation in bacterial-fungal consortia, enhances degradation efficiency. This occurs as intermediate metabolites produced by one strain can serve as substrates for the enzymatic catalysis of another (Tao et al., 2017; Abd El-Aziz et al., 2021; Espinosa-Ortiz; Rene; Gerlach, 2022). Consequently, the exploration and characterization of novel strains with potential applications in bioremediation, along with the

strategic formulation of microbial consortia by selecting species with desirable traits, may lead to promising scientific and economic prospects.

Building upon these insights, this review provides a comprehensive examination of microbial species involved in the biodegradation of polycyclic aromatic hydrocarbons (PAHs), with a particular emphasis on the application of microbial consortia and the synergistic interactions that drive the biotransformation of these pollutants. This review will explore some of the key enzymatic and metabolic mechanisms underlying PAHs degradation, highlighting the role of biosurfactant production, co-metabolism, and complementary enzymatic pathways in enhancing bioremediation efficiency. Furthermore, it will discuss strategic methodologies for selecting competent microbial strains, including advanced screening techniques and compatibility assessments, to optimize consortium formulation. By offering a structured framework for microbial strain prospecting, this review aims to contribute to the development of innovative and sustainable bioremediation solutions for PAHs-contaminated environments.

3.2. Insights into Bacterial Hydrocarbon Degradation Mechanisms

As notable decomposers of organic matter, numerous species of bacteria are recognized for their central role in the cycling and degradation of petroleum-derived pollutants (Maier; Gentry, 2014; Ferreira; Varjani; Taherzadeh, 2020). The emergence of such specialized species is intrinsically linked to the environmental modulation exerted by contaminants (Ghosal et al., 2016; Jayasena; Perera, 2021). Bacterial species such as *Alkanindiges sp.*, previously rare in soil, became predominant following diesel oil contamination, exemplifying the resilience and restructuring of local microbial communities in response to such disturbances (Fuentes et al., 2016). Similarly, obligate hydrocarbonoclastic species such as *Alcanivorax*, *Marinobacter*, *Thalassolituus*, *Cycloclasticus*, *Oleispira*, among others, which were either of low abundance or undetectable prior to petroleum pollution, were found to dominate after the impact of these contaminants (Yakimov; Timmis; Golyshin, 2007).

Associated with this metabolic flexibility, certain species employ a variety of mechanisms, often involving the production of intra- and extracellular enzymes, including monooxygenases, dioxygenases, laccases, peroxidases, hydroxylases, and dehydrogenases (Brzeszcz; Kaszycki, 2018; Imam et al., 2022). Among these enzymes involved in hydrocarbon degradation, monooxygenases are described as key mediators in the aerobic breakdown of

aliphatic hydrocarbons by bacterial species, initiating the process by oxidizing alkanes to alcohols (Rojo, 2009; Pandey; Pathak; Dave, 2016). This is followed by further oxidation steps, ultimately leading to fatty acids that enter the β-oxidation pathway (Abbasian et al., 2015). Various types of alkane hydroxylases exist, including AlkB-like monooxygenases, soluble di-iron monooxygenases, and cytochrome P450 enzymes (Van Beilen; Funhoff, 2007; Rojo, 2009). These enzymes differ in their genetic organization, regulatory mechanisms, and substrate ranges (Fenibo et al., 2023).

Bacterial dioxygenases and monooxygenases, also play a crucial role in the degradation of more complex hydrocarbons, such as polycyclic aromatic hydrocarbons (PAHs), by initiating the biochemical attack on the aromatic rings and converting them into dihydroxylated intermediates (Sierra-Garcia et al., 2013). These intermediates are then processed via ortho- or meta-cleavage, generating simpler molecules that can enter the tricarboxylic acid (TCA) cycle (Cerniglia, 1992; Mallick; Chakraborty; Dutta, 2011). However, the inherent complexity and low solubility of PAHs often limit bacterial degradation to partial breakdown or co-metabolic processes, as these properties reduce the bioavailability of PAHs to microbial enzymes (Husain, 2008; Seo; Keum; Li, 2009). Despite these challenges, some bacterial species have demonstrated the ability to mineralize polycyclic aromatic hydrocarbons (PAHs), including high-molecular-weight PAHs. *Bacillus thuringiensis* NA2 species isolated from contaminated sediments has shown promise in degrading high-molecular-weight PAHs, such as fluoranthene and pyrene (Maiti; Das; Bhattacharyya, 2012). Additionally, species such as *Sphingobium indicum*, *Sphingobium japonicum*, and *Stenotrophomonas maltophilia* have exhibited high efficiency in the degradation of polycyclic aromatic hydrocarbons (PAHs), including anthracene, benzo[a]anthracene, and benzo[a]pyrene (Gurjeet et al., 2014).

Recent studies have further expanded our understanding of the diversity and metabolic capabilities of hydrocarbon-degrading bacteria, identifying over 30 genera capable of degrading hydrocarbons. Among these, species from the genera *Pseudomonas*, *Bacillus*, *Stenotrophomonas*, *Ochrobactrum*, and *Rhodococcus*, isolated from the same region in Saudi Arabia, have demonstrated the ability to degrade both simple and complex PAHs, including benzo[a]pyrene and coronene (Mordecai et al., 2024). Other representative genera associated with the degradation of petroleum-derived hydrocarbons include *Achromobacter*, *Acinetobacter*, *Alkanindiges*, *Alteromonas*, *Enterobacter*, *Arthrobacter*, *Burkholderia*, *Dietzia*, *Kocuria*, *Marinobacter*, *Mycobacterium*, *Pandoraea*, *Staphylococcus*, and *Serratia*, among others, as detailed in Table 1 (Ramos et al., 2014; Basim et al., 2022; Chicca et al., 2022).

Table 1. Bacterial Species Involved in Hydrocarbon Biodegradation and Their Environmental Origins.

Species	Source	Hydrocarbons	Degradation (%)	Reference
Bacillus subtilis	River sediments contaminated with hydrocarbons (Nigeria)	Crude oil Pyrene	94% 85%	(Oyetibo et al., 2017)
Stenotrophomonas maltophilia	Soil contaminated with hydrocarbons (Australia)	Pyrene Fluoranthene Benzo[a]anthracene Benzo[a]pyrene Dibenz[a,h]anthraces Coronene	98% 45% 26% 22% 22% 55%	(Juhasz; Stanley; Britz, 2000)
Sphingomonas paucimobilis	Soil contaminated with oil (China)	Phenanthrene	98.7%	(Xia et al., 2005)
Serratia sp.	Roots contaminated with PAH (China)	Pyrene	51.2%	(Zhu et al., 2017)
Pseudomonas sp.	Gasoline enrichment (Mexico)	Benzene Toluene Ethylbenzene Xylene	90% 90% 90% 90%	(Morlett-Chávez et al., 2010)
Pseudomonas aeruginosa *Acinetobacter lwoffii* *Bacillus cereus*	Oil production wells (China)	n-alkanes	98.2% 29.90% 98.8%	(Liu et al., 2020)
Microbacterium steraromaticum *Bacillus infantis*	Soil from contaminated wells (Canada)	Benzene Toluene Ethylbenzene Xylene	67.98 % 72.8% 77.52% 74.58%	(Kaur et al., 2023)
Serratia marcescens	Soil contaminated with oil (China)	n-alkanes Phenanthrene Pyrene	66.17% 77.48% 32.89%	(Zhang et al., 2021)
Acinetobacter vivianii	Soil contaminated with oil (China)	Diesel	40%	(Zhang et al., 2022)
Rhodococcus sp.	Activated sludge obtained from wastewater treatment plant (China)	o-xylene	59%	(You et al., 2018)
Pseudomonas sp.	Estuarine sediment (Thailand)	Benzene Toluene Ethylbenzene Xylene	99% 99% 86% 82%	(Khodaei et al., 2017)
Achromobacter xylosoxidans	Soil contaminated with oil (China)	Fluoranthene	92.8 %	(Ma et al., 2015)

Ochrobactrum anthropic *Fusarium* sp.	Soil contaminated with hydrocarbons (Mexico)	Fluoranthene Pyrene	50.39% 51.32% 49.85% 49.36%	(Ortega-González et al., 2015)
Mycobacterium cosmeticum	Soil contaminated with hydrocarbons (China)	Benzene Toluene Ethylbenzene Xylene	99.90% 99.90% 99.90% 99.90%	(Zhang et al., 2013)
Campylobacter hominis *Bacillus cereus* *Dyadobacter koreensis* *Pseudomonas aeruginosa* *Micrococcus luteus*	Bitumen sediments (Africa)	PAHs	56% 66% 60% 59% 58%	(O. Olowomofe et al., 2019)
Mycobacterium sp.	River sediments (Korea)	Pyrene	99%	(Kim et al., 2018)
Mycobacterium sp.	Soil contaminated with PAH (China)	Phenanthrene	100 %	(Zeng et al., 2010)
Comamonas testosteroni	Isolate bank (China)	PAHs	60%	(Jiang et al., 2023)
Amycolatopsis sp.	Soil contaminated with oil (Mexico)	Naphthalene Anthracene Pyrene Fluoranthene	100% 37.9% 25.1% 18%	(Ortega-Gonzalez et al., 2015)
Pseudomonas aeruginosa	Soil contaminated with hydrocarbons (Mexico)	Total petroleum hydrocarbons	80%	(Lázaro-Mass et al., 2023)

3.3. Insights into Fungal Hydrocarbon Degradation Mechanisms

In parallel with the cycling of hydrocarbons, the involvement of fungal species has proven crucial for the complete mineralization of these contaminants, primarily due to their ability to secrete a diverse array of oxidative enzymes, such as laccases and peroxidases (Daccò et al., 2020a; Ferreira; Varjani; Taherzadeh, 2020). The products of this enzymatic activity can, in turn, benefit numerous other microbial species in the environment. Which, through co-metabolic process, contribute to the complete removal of various xenobiotic compounds (Liu et al., 2017; Chen et al., 2023). Another beneficial mechanism exhibited by some filamentous species is their vegetative propagation in soil through hyphal elongation, which is recognized for forming extensive network systems (Harms; Schlosser; Wick, 2011). This peculiar mode of

expansion offers several advantages for hydrocarbon biodegradation, including the transport or translocation of water and essential nutrients over long distances, allowing fungi to survive under stressful conditions and access hydrocarbons dispersed across various heterogeneous matrices (Harms; Schlosser; Wick, 2011; Furuno et al., 2012).

Considering these characteristics, the growth of certain fungi in alkane mixtures has been recognized since the early 20th century (Zobell, 1946). At that time, the most commonly reported species were ascomycetes belonging to the genera *Penicillium* and *Aspergillus*. Since then, a wide diversity of hydrocarbonoclastic fungal genera has been reported in contaminated environments, including species from *Trichoderma*, *Pleurotus, Amorphoteca*, *Neosartorya, Talaromyces, Fusarium, Paecilomyces, Sporobolomyces, Cephalosporium,* and *Graphium,* among others, as listed in Table 2 (Samuels et al., 2006; Barnes et al., 2018; Vipotnik; Michelin; Tavares, 2022).

Table 2. Fungal Species Involved in Hydrocarbon Biodegradation and Their Environmental Origins.

Species	**Source**	**Hydrocarbons**	**Degradation (%)**	**Reference**
Trametes versicolor	Soil contaminated with hydrocarbons (Nigeria)	Chrysene Benzo[a]pyrene	91% 49,1%	(Vipotnik; Michelin; Tavares, 2022)
Lasiodiplodia theobromae	Soil contaminated with PAHs (China)	Benzo[a]pyrene	53%	(Cao et al., 2020)
Tolypocladium sp.	Marine sponges (Brazil)	Pyrene	95%	(Vasconcelos et al., 2019)
Penicillium janthinellum *Aspergillus terreus* *Penicillium decumbens*	Soil contaminated with petrochemicals (India)	Kerosene	95% 96% 75%	(Khan; Nirmal Kumar; Nirmal Kumar, 2015)
Penicillium oxalicum	Lagoon contaminated with petrochemicals (Spain)	Anthracene Dibenzothiophene Phenanthrene Dibenzofuran	99% 99% 99% 99%	(Aranda et al., 2017)
Trichoderma longibrachiatum *Byssochlamys spectabilis*	Laboratory waste contaminated with petrochemicals	Benzo[a]anthracene	97% 79%	(Rosales et al., 2012)
Fusarium ateritium *Drechslera* sp. *Papulaspora* sp.	Desert (Kuwait)	Crude oil	71.6% 94.8% 93.1%	(Obuekwe et al., 2005)
Marasmiellus sp.	Marine sponges (Brazil)	Pyrene Benzo[a]pyrene	92.8% 97.2%	(Vieira et al., 2018)

Paecilomyces sp.	Soil contaminated with petrochemicals (South Korea)	Anthracene Phenanthrene Benzo[a]anthracene Benzo[b]fluoranthene	88% 75% 67.5% 99.3%	(Velmurugan et al., 2017)
Coriolopsis byrsina	Wood fragments (India)	Pyrene	96%	(Agrawal; Shahi, 2017)
Anthracophyllum discolor	Wood fragments (Chile)	Phenanthrene Anthracene Fluoranthene Pyrene Benzo[a]pyrene	62% 73% 54% 60% 75%	(Acevedo et al., 2011)
Ganoderma lucidum	Wood fragments (India)	Phenanthrene Pyrene	99.65% 99.58%	(Agrawal; Verma; Shahi, 2018)
Fusarium sp.	Sand contaminated with petrochemicals (Japan)	n-Octadecane	89%	(Hidayat; Tachibana, 2013)
Penicillium simplicissimum *P. janthinellum* *Phialophora alba* *P. hoffmannii* *Trichoderma harzianum* *Gliocladium virens*	Sediments contaminated with PAHs (France)	Pyrene	80%	(Ravelet et al., 2000)
P. simplicissimum *P. chrysosporium* *Irpex lacteus*	Mycobank (Spain)	Anthracene	86% 40% 38%	(Jove et al., 2016)
Aspergillus terreus	Soil contaminated with petrochemicals (Egypt)	Anthracene Naphthalene	91% 98.5%	(Mohamed I. A. Ali, 2012)
Aspergillus sydowii	Mangrove soil (Nigeria)	Anthracene	98,70%	(Bankole et al., 2020)

In the degradation of aliphatic hydrocarbons, non-ligninolytic species, predominantly from the phylum *Ascomycota*, employ metabolic pathways that involve the oxidation via cytochrome P450 monooxygenases (CYP) (Xue; Warshawsky, 2005; Al-Turki, 2009). These enzymes catalyze the initial hydroxylation of n-alkanes, converting them into alcohols, which are subsequently oxidized to form aldehydes and fatty acids. These fatty acids are then integrated into central metabolic pathways, undergoing β-oxidation before being further metabolized in the tricarboxylic acid cycle (Van Beilen; Funhoff, 2007; Prenafeta-Boldú; De Hoog; Summerbell, 2019). In addition to their established role in the degradation of aliphatic hydrocarbons, fungi have also demonstrated significant potential in the breakdown of PAHs. These complex compounds are often degraded via three primary enzymatic mechanisms: intracellular oxidation by CYP, non-specific oxidative action mediated by secreted ligninolytic peroxidases, and the activity of extracellular laccases (Haritash; Kaushik, 2009; Pozdnyakova

et al., 2018). For example, *Aspergillus sydowii* has been shown to degrade benzo[a]pyrene (BaP) and phenanthrene under hypersaline conditions, utilizing CYP-mediated pathways that result in the formation of less toxic metabolites (Peidro-Guzmán et al., 2021). Similarly, *Fusarium solani* has been reported to metabolize BaP through oxidative mechanisms involving hydrogen peroxide and reactive oxygen species, highlighting an alternative pathway for PAH transformation (Veignie et al., 2004). Furthermore, ligninolytic fungi such as *Pleurotus ostreatus* employ extracellular peroxidases and laccases to break down PAHs like anthracene and pyrene, effectively reducing their environmental toxicity and promoting mineralization into carbon dioxide (Kadri et al., 2017). White-rot fungi, including *Phanerochaete chrysosporium*, have demonstrated high efficiency in the degradation of high-molecular-weight PAHs by utilizing a combination of ligninolytic and non-ligninolytic enzymes, achieving significant degradation rates even under challenging environmental conditions (Boonchan; Britz; Stanley, 2000).

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3.4. Strategies for Identifying and Isolating Hydrocarbonoclastic Microorganisms

In this context, hydrocarbon-contaminated environments have proven to be a rich matrix for the prospecting of microbial species capable of degrading these pollutants (Pandolfo; Barra Caracciolo; Rolando, 2023). To this end, well-designed screening strategies that integrate microbiological, biochemical, and molecular methods are essential to rapidly and accurately identify competent species (Lee et al., 2020; Jia et al., 2023). Once native strains with desirable metabolic traits for hydrocarbon degradation are identified, their potential application in bioremediation can be further explored. As suggested by Tao et al. (2017), these microorganisms can be cultivated in the laboratory and subsequently reintroduced into their original environment, a strategy known as bioaugmentation. This approach offers advantages over the use of exogenous microorganisms, as native isolates are more likely to reestablish and proliferate effectively in their natural habitat, enhancing biodegradation efficiency (Nwankwegu et al., 2022). In a practical application, bacterial strains of *Rhodococcus erythropolis* CD 130 and CD 167, previously isolated from petroleum-contaminated soil, were reintroduced into their native environment. Functional metagenomic analysis of the treated soil microbiome revealed that the reintroduced native bacteria played a critical role in the initial stages of degradation, achieving total hydrocarbon removal efficiencies of 38.40% and 29.80%, respectively. In contrast, untreated soil exhibited a significantly lower removal efficiency of only 14.77% (Pacwa-Płociniczak et al., 2020). In a separate study, bioaugmentation with

indigenous *Acinetobacter tandoii* led to a substantial increase in phenanthrene degradation in PAH-contaminated wastewater, with alterations in the microbial community diversity that favored the emergence of new hydrocarbon degraders (Li et al., 2018). Similarly, the combined application of bioaugmentation and biostimulation, along with nutrient supplementation, has demonstrated significant potential for enhancing hydrocarbon degradation in aquatic environments. For instance, in petroleum-contaminated mesocosms, the use of native microbial consortia, including *Bacillus subtilis*, resulted in a 75% reduction in total petroleum hydrocarbons (TPH) (Dellagnezze et al., 2016).

From this perspective, the isolation of hydrocarbonoclastic strains can be accomplished through targeted screening techniques. One such methodology involves the isolation of microorganisms using minimal media, such as Bushnell-Haas (BH) and Minimal Salt Medium (MSM), where the contaminant hydrocarbon serves as the sole carbon source (Bushnell; Haas, 1941; Hilyard et al., 2008). This enrichment strategy operates under the premise that only microorganisms capable of metabolizing the pollutant will be able to grow and proliferate, facilitating the identification of strains with degradative potential (Shao, 2010). By applying this methodology Tripathi et al. (2023), successfully isolated hydrocarbon-degrading strains from petroleum-contaminated soil samples, including *Pseudomonas boreopolis* IITR108, *Microbacterium schleiferi* IITR109, *Pseudomonas aeruginosa* IITR110, and *Bacillus velezensis* IITR111. These strains demonstrated the ability to degrade both aliphatic (C8–C40) and aromatic hydrocarbons. Beyond bacteria, numerous hydrocarbonoclastic fungal species belonging to the genera *Aspergillus, Trichoderma,* and *Penicillium*, among others, have also been obtained using this approach (Dhar; Dutta; Anwar, 2014; Benguenab; Chibani, 2020; Daccò et al., 2020b; Taha et al., 2020).

Alongside the initial isolation phase, complementary analyses can be employed to refine the selection of relevant strains by providing a more comprehensive assessment of their hydrocarbon degradation potential. These include quantifying fungal biomass accumulation during growth in liquid media enriched with hydrocarbon source (Bovio et al., 2017; Rani et al., 2024), evaluating tolerance to varying hydrocarbon concentrations (Dos Santos et al., 2008; Zafra et al., 2015), and assessing growth rate and colony diameter in hydrocarbon-exposed cultures on solid media (Argumedo-Delira et al., 2012; Benguenab; Chibani, 2020). Additionally, bacterial growth kinetics can be monitored through optical density measurements (OD600), offering valuable insights into adaptability and potential efficacy in hydrocarbon consumption (Koolivand et al., 2019; Wu et al., 2021).

Another simple method that can facilitate the rapid assessment of the degradative potential of microbial isolates is the use of the redox indicator 2,6-dichlorophenolindophenol (DCPIP) in enriched liquid cultures (Hanson; Desai; Desai, 1993). In this colorimetric assay, DCPIP is incorporated by microorganisms capable of hydrocarbon degradation, acting as an electron acceptor and transitioning from blue (oxidized) to colorless (reduced). Thus, the decolorization of the culture serves as a direct indicator of the metabolic activity of microorganisms utilizing the available carbon source (Montagnolli; Lopes; Bidoia, 2015).

The following figure provides a schematic representation of these initial steps, illustrating key analyses that contribute to the screening of strains with potential for petroleum hydrocarbon bioremediation.

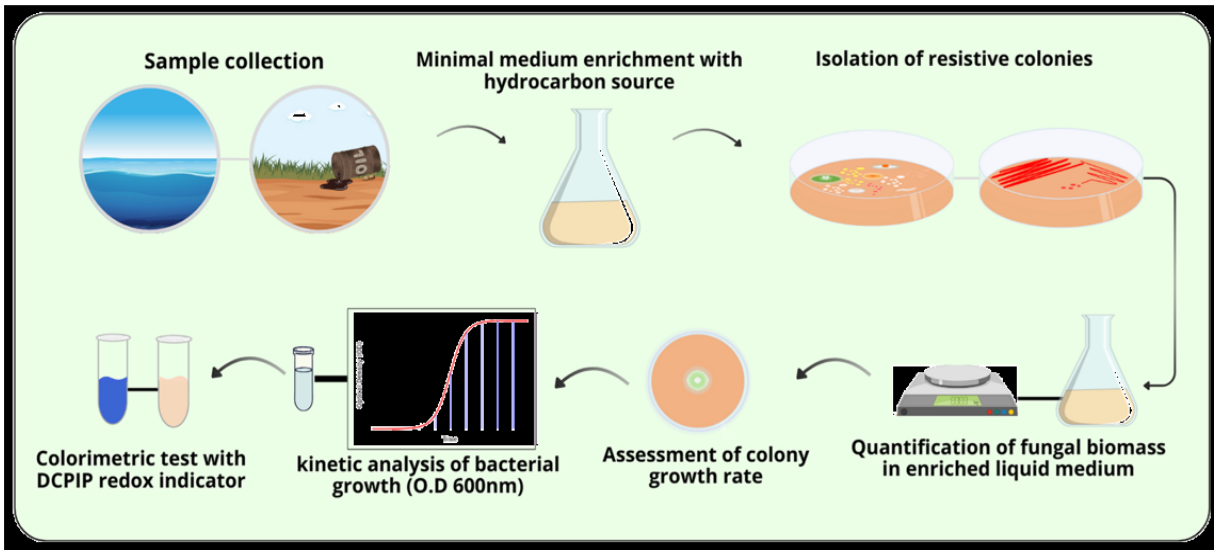

Figure 1. Stepwise framework for screening and selecting hydrocarbon-degrading microorganisms.

3.5. The Role of Microbial Biosurfactants in PAHs Biodegradation and Screening Approaches

However, biodegradation can become challenging depending on the concentration and nature of the hydrocarbon present, requiring local microbial communities to adopt alternative mechanisms to overcome the limitations imposed by the contaminant substrate (Aydin et al., 2017; Pacwa-Płociniczak et al., 2020). This scenario is particularly observed in hydrocarbons resistant to biochemical degradation, such as PAHs, which, due to their high hydrophobicity and the presence of two or more fused aromatic rings, exhibit low bioavailability (Bosma et al., 1997; Ghosal et al., 2016).

In response to the presence of these hydrocarbons, the production of biosurfactants by certain fungal and bacterial strains has been shown to play a crucial role in increasing bioavailability, thereby facilitating subsequent biodegradation (Chandankere et al., 2014; Da Silva et al., 2021). This effect is primarily attributed to the ability of these molecules to reduce surface and interfacial tension, thereby expanding the contact area of the hydrophobic portions of the contaminants and enhancing their (Almansoory et al., 2019). Considering these aspects, the adoption of methodologies focused on detecting and quantifying biosurfactants produced by native microorganisms from impacted environments can significantly contribute to the selection of microbial strains directly involved in the transformation and degradation of persistent hydrocarbons (Walter; Syldatk; Hausmann, 2010; Sharma; Lavania; Lal, 2022).

Various methods have been developed to quantify surface tension reduction resulting from the action of biosurfactants, each offering specific advantages. The Wilhelmy plate method and the Du Noüy ring method are widely used for equilibrium measurements, both relying on the interaction of a solid probe with the liquid surface to quantify the tension forces (Bakhshi; Soleimanian-Zad; Sheikh-Zeinoddin, 2017). An alternative and simple method is the drop shape analysis, which evaluates the curvature of a liquid droplet in contact with a hydrophobic surface, providing insights into the wettability and spreading properties of biosurfactant solutions (Van Der Vegt et al., 1991). Similarly, the oil spreading assay measures the reduction in interfacial tension between aqueous and oil phases, where biosurfactants destabilize intermolecular forces, increasing the spread area of the oil layer (Youssef et al., 2004; Archana et al., 2019). Another widely used screening method is the drop-collapse test, a simple and rapid technique for detecting biosurfactants. The presence of a biosurfactant reduces the droplet's surface tension, causing it to collapse. This disturbance in the droplet's shape can be quantified using a goniometer, allowing for comparative analysis against control samples lacking biosurfactants (Bodour; Miller-Maier, 1998; Woźniak-Karczewska et al., 2017).

For primary screening of biosurfactant-producing strains**,** methylene blue-CTAB agar can be employed to detect extracellular anionic biosurfactants. This method is based on the formation of halos around microbial colonies due to interactions with the cationic detergent CTAB, providing a straightforward visual identification of biosurfactant production (Siegmund; Wagner, 1991; Eldin; Kamel; Hossam, 2019). Another qualitative approach is the blood hemolysis test, where biosurfactants interact with erythrocytes present in the medium,

causing cell lysis. This technique is particularly useful for detecting specific classes of biosurfactants such as rhamnolipids (Mulligan; Cooper; Neufeld, 1984; Marcelino et al., 2019).

High-throughput approaches, such as microplate assays and penetration tests, enable the efficient analysis of a large number of isolates while minimizing sample consumption (Da Silva et al., 2021). The microplate assay detects biosurfactants based on optical distortion caused by changes in surface tension, whereas the penetration test, a colorimetric technique, identifies biosurfactant presence through color changes resulting from interactions between two immiscible phases (Walter; Syldatk; Hausmann, 2010; Mnif; Ghribi, 2015; Mohanram; Jagtap; Kumar, 2016). Conversely, the emulsification index (E24) quantifies the ability of biosurfactants to stabilize oil-water emulsions by measuring the height of the emulsion layer relative to the total liquid volume after 24 hours of incubation (Abouseoud et al., 2008; Da Silva et al., 2023).

Another key parameter that can be used to assess biosurfactant activity is the Microbial Adhesion to Hydrocarbons (MATH) assay, which evaluates cell surface hydrophobicity by measuring the differential migration of microbial cells between aqueous and hydrophobic phases. Since increased hydrophobicity is often associated with enhanced emulsification efficiency and improved assimilation of hydrophobic substrates, this method provides indirect evidence of biosurfactant production and its potential role in hydrocarbon biodegradation (Kaur; Sangwan; Kaur, 2017; Al-Hawash et al., 2018).

The application of this methodological framework in a practical study, including selective enrichment in minimal media, colorimetric assays using the redox indicator DCPIP, and biosurfactant production analysis through emulsification activity assays and surface tension quantification, enabled the identification of three outstanding fungal strains among the 15 initially isolated from crude oil-contaminated coastal soil samples: *Aspergillus niger* (Y1), *Aspergillus oryzae* (Y2), and *Penicillium commune* (Y4). Among these, *A. oryzae* (Y2) demonstrated the highest degradation efficiency, achieving approximately 99% crude oil removal. This result was directly linked to the strain's elevated biosurfactant production, which outperformed the other isolates, exhibiting an emulsification index (E.I24) of 75.6% and reducing the medium's surface tension to 23 mN/m (El-Hanafy et al., 2017). Similarly, Rehman et al. (2021) found that rhamnolipids and sophorolipids produced by strains of *Pseudomonas aeruginosa* and *Meyerozyma* sp. were effective in incorporating various crude oil constituents into their mixed micelles, achieving an E.I24 of 87% and 84%, respectively. This process

facilitated crude oil degradation, leading to the removal of 91% and 87% of the contaminant by the analyzed strains. Furthermore, the biosurfactants under study exhibited stability across a range of pH levels, temperatures, and NaCl concentrations, demonstrating strong potential for biotechnological applications. Displaying a similar resilience to environmental variations, the biosurfactant serrawetin W1, produced by the *Serratia marcescens* S-1 strain, enhanced the bioavailability of diesel oil in liquid media, achieving an E.I24 of 65.8%. Reinforcing the importance of these molecules in optimizing bioremediation processes, the strain exhibited degradation efficiencies of 67.3% for diesel oil, 40.59% and 20.27% for the PAHs phenanthrene and pyrene, respectively, and 82.38% for *n*-alkanes (Zhang et al., 2021). In summary, Figure 2 offers a clear and illustrative overview of the screening methods employed for the isolation and evaluation of biosurfactant-producing microorganisms.

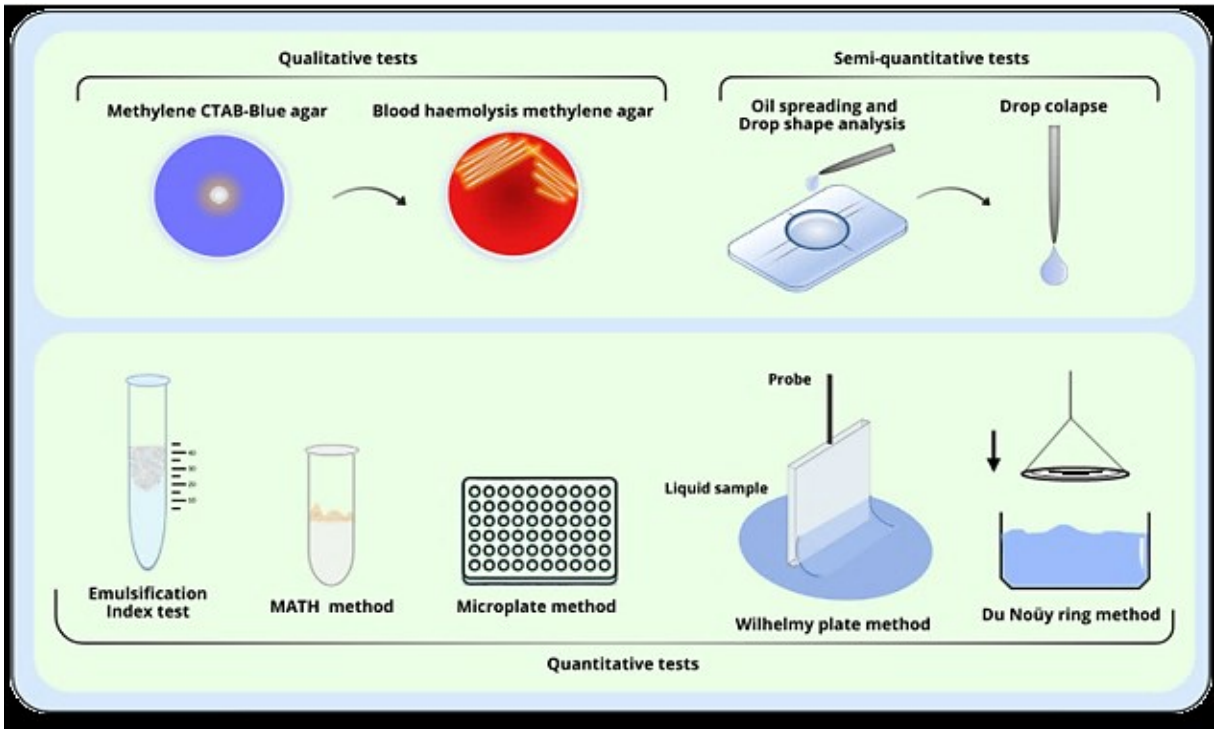

Figure 2. Overview of screening methods for the isolation and evaluation of biosurfactant-producing microorganisms.

An additional strategy explored by researchers involves the direct application of purified or concentrated biosurfactants, either *in situ* or in simplified artificial ecosystems known as "microcosms," to enhance the bioavailability of hydrophobic hydrocarbons (Li; Li, 2011; Vaishnavi et al., 2021). The supplementation of rhamnolipid JBR-425 in a liquid medium containing crude oil as the sole carbon source has been shown to significantly impact the

efficiency of PAHs degradation by a bacterial consortium composed of *Ochrobactrum anthropi* IITR07, *Pseudomonas mendocina* IITR46, *Microbacterium esteraromaticum* IITR47, *Pseudomonas aeruginosa* IITR48, and *Stenotrophomonas maltophilia* IITR87. In the presence of rhamnolipid, the consortium achieved degradation efficiencies of 97.3% for naphthalene, 76.2% for fluorene, and 96.5% for phenanthrene. Conversely, the absence of biosurfactant supplementation resulted in reduced contaminant bioavailability, which was reflected in lower degradation rates of 89.1% for naphthalene, 63.8% for fluorene, 81% for phenanthrene, and 72.8% for benzo(b)fluoranthene (Kumari; Regar; Manickam, 2018). A detailed analysis of the influence of biosurfactant supplementation on the metabolism of *Burkholderia xenovorans* during the degradation of polybrominated diphenyl ethers revealed that the presence of these molecules is correlated with a progressive accumulation of the contaminant within cells, facilitating pollutant uptake for biodegradation (Ti et al., 2020).

3.6. The Role of Oxidative Enzymes in PAHs Biodegradation and Screening Approaches

In addition to biosurfactant activity, the production of enzymes such as laccases (LaC, EC:1.10.3.2), manganese peroxidases (MnP, EC:1.11.1.13), lignin peroxidases (LiP, EC:1.11.1), and versatile peroxidases (VP, EC:1.11.1.16) has been shown to play a crucial role in the simultaneous oxidation of a wide range of aromatic compounds (Verdin; Sahraoui; Durand, 2004; Kadri et al., 2017). In PAHs degradation, for instance, laccases catalyze the removal of electrons from aromatic rings, generating free radicals that destabilize their structure. This process leads to the formation of intermediate products such as quinones, which are more reactive and susceptible to further transformations (Cerniglia, 1992; Gupte et al., 2016). A distinctive factor in this process, often associated with fungal species belonging to the genera *Trichoderma, Penicillium, Aspergillus, Trametes*, and *Pleurotus* is their ability to produce and secrete these enzymes into the extracellular matrix, thereby facilitating the initial biochemical attack on PAHs in heterogeneous environments (Pandey; Pathak; Dave, 2016; Pozdnyakova et al., 2018; Zehra et al., 2018; Ma et al., 2021). Al-Hawash, Zhang e Ma (2019) support this observation by highlighting that the extracellular enzymes of *Aspergillus* sp. RFC-1 were more effective in the degradation of various hydrocarbons, including naphthalene and pyrene, compared to intracellular enzymes. The researchers also linked the production of biosurfactants and the assimilation of contaminants by the strain's biomass to enhanced degradation efficiencies of 51.8% for crude oil, 84.6% for naphthalene, 50.3% for

phenanthrene, and 55.1% for pyrene. These findings suggest that utilizing strains equipped with distinct degradation mechanisms may be a more effective approach in bioremediation strategies.

A molecular analysis of the fungal strains *Aspergillus flavus* AF15, *Trichoderma harzianum* TH07, *Fusarium solani* FS12, and *Aspergillus terreus* revealed that the high expression of genes encoding laccases (*lcc1u*), lignin peroxidase (*lig1, lig2, lig4, lig6*), and manganese peroxidase (*mnp1u*) was a key factor in the adaptability and growth of these strains under high crude oil concentrations (20%) (Al-Zaban; AlHarbi; Mahmoud, 2021). Similarly, when investigating the metabolic mechanisms of *F. solani*, isolated from PAH-contaminated mangrove sediments, Wu, Luo e Vrijmoed (2010) reported that the strain was capable of degrading two specific PAHs: anthracene (40%) and benzo[a]anthracene (60%). Their findings suggest that PAH degradation occurs through aromatic ring oxidation into quinones, followed by phthalic acid formation, with extracellular laccases playing a key role in contaminant conversion.

Among the different approaches for screening enzyme-producing strains, colorimetric techniques remain one of the most commonly employed (Lee et al., 2020). For rapid and simple detection in primary screening stages, the Bavendamm test, for example, assesses the oxidation capacity of strains in a medium enriched with gallic or tannic acid (Lindeberg, 1948; Leonard, 1971; Shleev et al., 2004). A color change in the agar plates to brown indicates the oxidation of these phenolic compounds, which is linked to the production of enzymes from the ligninolytic complex. Similarly, the use of guaiacol in malt extract agar (MEA) medium supplemented at 0.2% (v/v) enables the qualitative identification of laccase and peroxidase production by fungal strains, as indicated by the oxidation of guaiacol, which results in a reddish-brown coloration (Kiiskinen; Rättö; Kruus, 2004; Kumar; Rapheal, 2011). An additional qualitative method that can be applied in secondary screening stages is the Remazol Brilliant Blue R (RBBR) decolorization assay. This test is characterized by the transition of the dye color from blue to a white-yellowish hue, signaling extracellular laccase activity (Pasti; Crawford, 1991; Palmieri; Cennamo; Sannia, 2005).

For quantitative analysis, one of the most widely used methods for assessing laccase and total peroxidase activity involves the oxidation of the substrate 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)**,** which acts as a redox mediator (Maryskova et al., 2021). This reaction is facilitated by crude enzymatic extracts obtained from microbial

liquid cultures, where, ABTS in the presence of these enzymes is oxidized into its radical cation $ABTS^{+}$, producing a characteristic green-blue coloration (Bourbonnais et al., 1995; Agrawal; Shankar; Verma, 2020). The increase in absorbance can be measured using a spectrophotometer at 420 nm for laccases and 412 nm for total peroxidases**,** providing a reliable indicator of enzymatic activity (Hunter et al., 2003; Eichlerová; Šnajdr; Baldrian, 2012). Complementary analytical methods enhance the specificity and accuracy of enzymatic quantification by utilizing alternative substrates. The oxidation of syringaldazine is frequently employed to determine laccase activity, as this substrate provides high sensitivity and specificity for this enzyme class (Grassin; Dubourdieu, 1989; Agrawal; Verma, 2019). Studies have demonstrated that different enzymatic substrates exhibit varying affinities depending on enzyme type and source. For instance, Neelkant et al. (2020) characterized a laccase from *Sphingobacterium ksn-11*, revealing that ABTS was the preferred substrate over syringaldazine and guaiacol, as indicated by its higher Vmax and Km values. Similarly, Matsumoto et al. (2020) compared the oxidation and polymerization capabilities of laccase from *Rhus vernicifera* and horseradish peroxidase, showing that laccase displayed greater activity towards syringaldazine, while peroxidase exhibited higher activity when using ABTS as a substrate. Another substrate employed for quantifying enzymatic activity is 2,6-dimethoxyphenol (DMP), which is commonly used to measure manganese peroxidase (MnP) activity due to its ability to act as a phenolic electron donor in oxidation reactions (Heinfling et al., 1998; Qin et al., 2018). In contrast, veratryl alcohol is a key substrate for assessing lignin peroxidase (LiP) activity, as its oxidation to veratraldehyde is a hallmark of LiP's catalytic function (Tien; Kirk, 1988; Vyas et al., 1994).

Adopting a screening framework similar to the key tests previously described for the prospecting and evaluation of enzyme-producing species within the ligninolytic complex, Lee et al. (2020) assessed a total of 120 fungal strains. The proposed protocol included the Bavendamm assay for detecting oxidative activity, the Remazol Brilliant Blue R (RBBR) decolorization test to evaluate laccase activity, and specific assays to assess tolerance to four selected PAHs: phenanthrene, anthracene, fluoranthene, and pyrene. This three-stage screening protocol enabled the selection of 14 white-rot fungal (WRF) strains with PAH degradation potential, including *Microporus vernicipes*, *Peniophora incarnata*, *Perenniporia subacida*, *Phanerochaete sordida*, *Phlebia acerina*, and *Phlebia radiata*. Among these, *P. incarnata* exhibited the highest PAH degradation activity, ranging from 40% to over 90%, which was associated with the variable secretion of laccases, manganese peroxidase, and lignin peroxidase.

Furthermore, the expression of the *pilc1* and *pimp1* genes, encoding laccase and MnP, respectively, confirmed that PAH biodegradation in the selected fungal strains was mediated by extracellular enzymes.

In another study, Wang et al., (2022) investigated the potential of *Bacillus atrophaeus* laccase for the degradation of polycyclic aromatic hydrocarbons (PAHs). The researchers employed the ABTS oxidation assay to quantify laccase activity, demonstrating that the enzyme exhibited strong oxidative potential towards ABTS. Additionally, engineered modifications to the enzyme structure led to a significant enhancement in PAH degradation**,** particularly for high-molecular-weight compounds. Figure 3 provides a concise and illustrative summary of the key screening methodologies employed to evaluate oxidative enzymes involved in hydrocarbon biodegradation

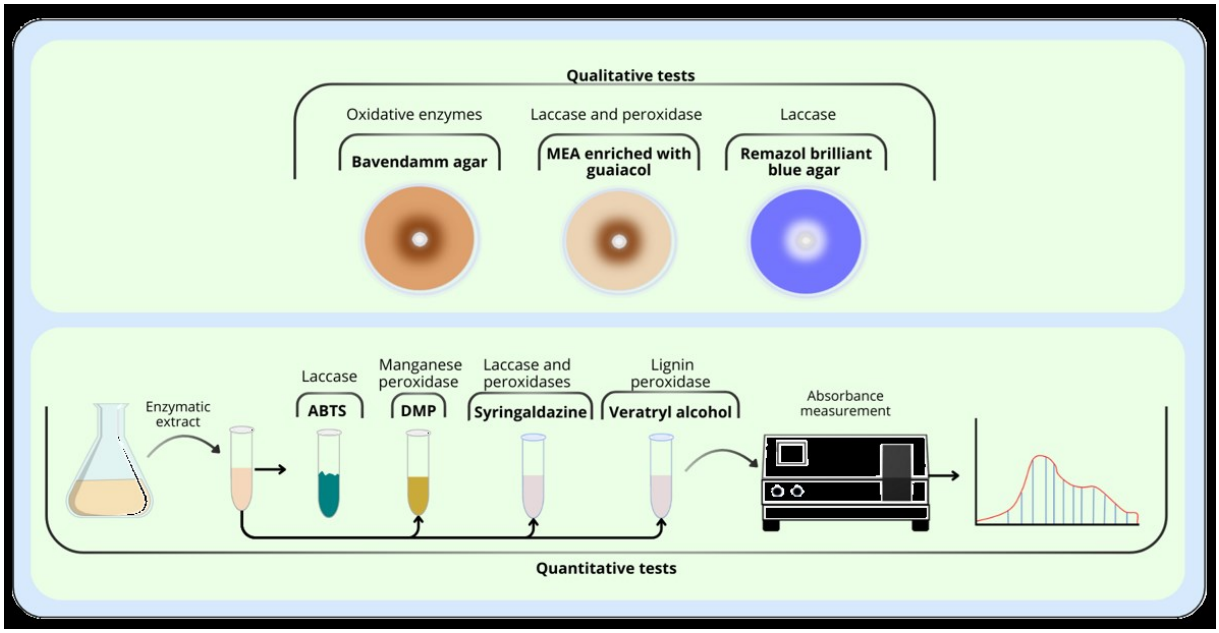

Figure 3. Overview of screening methodologies for the evaluation of oxidative enzymes involved in hydrocarbon biodegradation.

A distinct strategy to enhance the efficiency of free laccases involves their immobilization onto various material matrices, providing advantages such as improved operational stability, increased resistance to organic degradation, and enhanced tolerance to pH variations (Zhang et al., 2020). This method also stands out for its economic feasibility, as it allows for enzyme reuse in successive treatment cycles. For instance, the immobilization of laccases produced by *Trametes versicolor* onto Fe_3O_4 magnetic particles coated with chitosan enabled enzyme reuse over three catalytic cycles, achieving degradation efficiencies of 81.9% and 69.2% for anthracene and benzo[a]pyrene, respectively (Deng et al., 2022). In another

study, the immobilization of laccases derived from *Pseudomonas* strains URS-5, URS-6, URS-8, and *Rhodococcus* URS-10 onto modified polyimide aerogels was also effective for PAH biodegradation. These immobilized laccases retained 80% of their initial activity even after three assay cycles, degrading 75% of the anthracene present in contaminated water samples. In comparison, free enzymes degraded less than 60% of anthracene under the same conditions (Davoodi et al., 2023). In this context, bacterial laccases offer certain advantages over their fungal counterparts, as they are monomeric and do not require post-translational modifications, simplifying the immobilization process (Zhang et al., 2020).

3.7. The Role of Microbial Consortia in Hydrocarbon Biodegradation

Despite the well-documented role of enzymatic complexes and biosurfactants in enhancing hydrocarbon biodegradation, complete mineralization of the heterogeneous hydrocarbon fractions present in petroleum remains a significant challenge (Chaerun et al., 2004; Shi; Guo, 2024). This can be attributed to the fact that a single microorganism typically metabolizes only a limited range of hydrocarbons, resulting in a lack of interaction with certain compounds or restricting its activity to partial degradation (Alexander, 1999; Tao et al., 2017). In the case of PAHs, certain biotransformation processes can lead to the generation of even more toxic intermediates, a phenomenon known as “biomagnification” (Cerniglia; Sutherland, 2010). Analyses of hydrocarbon-contaminated environments, have highlighted the critical role of co-metabolic interactions exhibited by native microbial communities as a key pathway for the complete mineralization of persistent hydrocarbons (Bellino et al., 2019; Alsayegh; Al-Ghouti; Zouari, 2021; Imam et al., 2022). When applied to bioremediation, the development of synthetic and natural microbial consortia with distinct metabolic capabilities has emerged as a promising strategy to expand the range of degradable compounds, thereby minimizing the accumulation of persistent or toxic intermediates and significantly enhancing overall bioremediation efficiency (Li et al., 2021; Massot et al., 2022).

From this perspective, multiple studies have demonstrated the effectiveness of fungal consortia in petroleum hydrocarbon degradation, particularly due to their synergistic metabolic interactions and enhanced enzyme production. For instance, Ameen et al. (2016) reveled that a fungal consortium composed of *Alternaria alternata*, *Aspergillus terreus*, *Cladosporium sphaerospermum*, *Eupenicillium hirayamae*, and *Paecilomyces variotii*, isolated from

hydrocarbon-contaminated mangrove sediments, was capable of completely degrading the main hydrocarbons present in diesel oil**.** The authors attributed the synergistic effect of this fungal assembly to the production of ligninolytic enzymes**,** particularly laccases (Lac), lignin peroxidase (LiP), and manganese peroxidase (MnP)**,** which were significantly higher compared to individual strains evaluated separately. Similarly, the syntrophic interaction between fungal consortia FC1 (*Penicillium chrysogenum* and *Cladosporium brachyspora*) and FC2 (*Scopulariopsis brevicaulis* and *Scopulariopsis botryosum*) led to significantly higher degradation rates of high-molecular-weight PAHs compared to those achieved by individual isolates (Hamad et al., 2021). The presence of biosurfactant-producing strains within these consortia further contributed to enhanced hydrocarbon bioavailability, accelerating the degradation process.

In another study, the elevated production of peroxidases and laccases by fungal strains within the consortium containing *Penicillium chrysogenum*, *Mucor racemosus*, and *Lasiodiplodia theobromae* was associated with the degradation of branched hydrocarbon isoprenoids, such as pristane (C_{17}) and phytane (C_{18}), in HPA-contaminated soil (Balaji; Arulazhagan; Ebenezer, 2014). According to Pozdnyakova et al. (2018), the combined activity of laccases and versatile peroxidases expressed by *Pleurotus ostreatus* was responsible for the conversion of phenanthrene and anthracene into quinones. These quinones were subsequently integrated into basal metabolism, leading to the complete mineralization of these compounds. In contrast, the same authors demonstrated that *Agaricus bisporus*, which produced only laccases, accumulated quinone metabolites without further transforming them into additional degradation products. This finding suggests that the association of different eco-physiological fungal groups, equipped with multiple enzymatic complexes, can significantly influence the degradation pathways and ultimate fate of these contaminants.

In addition to fungi, the synergistic interactions within bacterial consortia have also demonstrated significant effectiveness in the removal of persistent hydrocarbons. For example, a bacterial consortium containing *Pseudomonas*, *Stenotrophomonas*, *Agrobacterium*, *Bacillus*, *Cupriavidus*, and *Trabulsiella* species was responsible for removal of 95% of high-molecular-weight (HMW) PAHs and 90% of low-molecular-weight (LMW) PAHs (Kuppusamy et al., 2016)**.** The authors also demonstrated that, in addition to their high efficiency in degrading complex hydrocarbons, the bacterial consortia were able to thrive under nutrient-limited conditions and tolerate acidic pH ranges. This finding suggest that when employed together,

these species exhibit enhanced adaptability to heterogeneous matrices. Poddar, Sarkar and Sarkar (2019), further emphasized this characteristic, noting the stress-resistant nature of a consortium composed of *Klebsiella* spp., *Enterobacter* sp., and *Pantoea* sp., which was responsible for the substantial degradation of mixed hydrocarbons.

In a 90-day intervention in crude oil-contaminated soil, Ebadi et al. (2021) demonstrated that bioaugmentation with a consortium consisting of three *Pseudomonas* strains, one *Bacillus* strain, and one *Chryseobacterium* strain resulted in the degradation of 51.6% of crude oil**,** 61.8% of aliphatic fractions, and 56.7% of aromatic fractions. Among the evaluated strains, biosurfactant production and high enzymatic activity, particularly of catechol 2,3-dioxygenase, were found to significantly influence the degradation process. Complementing these findings, Chen et al. (2023) reported that bacterial consortia derived from petroleum hydrocarbon-contaminated soil achieved a 31.54% higher degradation efficiency than individually applied strains, underscoring the significance of syntrophic interactions and the superior efficacy of microbial consortia in bioremediation strategies.

3.7.1. Enhancing PAHs Degradation through Mutualistic Fungal-Bacterial Consortia

Alongside the recognized bioremediation potential of separate fungal and bacterial consortia, mixed fungal-bacterial systems have increasingly captured scientific interest, driven by the frequent co-occurrence of these microbes in environments contaminated by petrochemicals (Husaini et al., 2008; Al-Turki, 2009; Ma et al., 2021). In such settings, interspecific interactions can yield synergistic effects where cooperative mechanisms prevail or antagonistic outcomes driven by nutrient competition and inhibitory metabolite production (Gorter; Manhart; Ackermann, 2020). For example, the expansive hyphal networks of filamentous fungi not only facilitate their own growth but also serve as “fungal highways” that enhance bacterial dispersal and colonization across porous matrices, thereby improving access to dispersed hydrocarbons (Wick et al., 2006; Warmink et al., 2011; Banitz et al., 2013). Research on hydrocarbon-degrading bacterial-fungal co-cultures has demonstrated that bacterial strains adhering to fungal surfaces benefit from improved substrate access and metabolic cooperation, ultimately achieving significantly higher hydrocarbon degradation rates compared to those observed in planktonic cultures (Utami et al., 2019). Moreover, the secretion of fungal exudates, such as glycerol, can stimulate bacterial activity, while bacteria in turn may

promote hyphal growth through the production of metabolites like auxofuran (Riedlinger et al., 2006; Nazir et al., 2013).

These cooperative interactions are particularly valuable in bioremediation processes, where co-cultures have demonstrated significant potential for the degradation of hydrocarbons via co-metabolic pathways (Kim; Lee, 2007; Martin et al., 2014). For instance, ligninolytic fungi contribute extracellular enzymes (e.g., laccases, peroxidases, and manganese peroxidases) that can initiate the oxidation of complex hydrocarbons, while bacteria metabolize the resulting degradation intermediates, thereby accelerating contaminant mineralization (Chávez-Gómez et al., 2003; Baldrian, 2004). This synergistic effect was observed during the degradation of BaP by a consortium composed of *Pleurotus ostreatus*, *Penicillium chrysogenum*, and *Pseudomonas aeruginosa*. The production of laccases by the fungi facilitates the initial oxidation phase of BaP, during which BaP-quinones are detected as intermediates. These intermediates are subsequently metabolized by the bacterial members of the consortium, ultimately resulting in a degradation rate of 86.1% (Bhattacharya et al., 2017). Moreover, Machín-Ramírez et al. (2010), reported that the synergistic interaction between strains of *Penicillium* sp. and *Serratia marcescens* was responsible for the removal of 65% of BaP.

Other mechanisms, such as biosurfactant production, have also been shown to play a role in these cooperative interactions (Kamyabi; Nouri; Moghimi, 2017). As an example, a study by Atakpa et al. (2022) demonstrated that a consortium comprising *Scedosporium* sp. and *Acinetobacter* sp. increased its biosurfactant production, which correlated with an improvement in the degradation rate of total hydrocarbons from 23.36% to 58.61% over 7 days. Furthermore, Ghorbannezhad, Moghimi and Dastgheib (2021) found that the addition of rhamnolipids significantly enhanced the degradation of pyrene and tetracosane. Conversely, Li and Li (2011) suggest that the combined use of more than one type of biosurfactant, such as rhamnolipids and sophorolipids, may yield an even greater synergistic effect in fungal-bacterial co-cultures, ultimately resulting in enhanced overall degradation of total hydrocarbons.

3.7.2 Screening Strategies for Compatible Strains and Consortium Formulation

However, ensuring compatibility among consortium members is critical, as antagonistic interactions arising from nutrient competition and the production of inhibitory metabolites can directly affect contaminant degradation rates (Wu et al., 2021). For example, while *Fusarium*

solani and *Arthrobacter oxydans* individually demonstrated the capacity to degrade phenanthrene, pyrene, and dibenzo[a,h]anthracene, their co-culture led to pH fluctuations and intensified nutrient competition. These adverse conditions ultimately inhibited the metabolic efficiency of the strains, resulting in reduced degradation rates of PAHs (Thion et al., 2012; Partovinia et al., 2023). Various in vitro methodologies have been developed to assess these interactions, thereby facilitating the identification and selection of microbial partners best suited for synergistic degradation processes. One widely used technique is the dual culture plate assay**,** which involves co-inoculating microbial strains onto agar plates and assessing growth inhibition zones, as illustrated in Figure 4**.** This method provides a straightforward means to detect competitive exclusion, metabolic interference, or antibiotic production among potential consortium members (Anith; Nysanth; Natarajan, 2021). Modified agar plate assays specifically designed to screen for antagonism among hydrocarbon-degrading strains have also been developed. These assays employ basal media such as Toyama or MSM, supplemented with hydrocarbons as the sole carbon source, to accurately assess inter-strain interactions under conditions that closely mimic contaminated environments (Wunder et al., 1994; Patowary et al., 2016).

One alternative method to assess antagonism between bacteria and fungi involves a modified agar plate assay, as described by Zafra et al. (2017). In this protocol, fungal strains are individually inoculated at the center of agar plates and incubated at 28 °C for four days to facilitate initial colony development. Once the fungal colonies have partially established, six bacterial strains are streaked from the periphery toward the center of the plate. The plates are then re-incubated at 28 °C until any antagonistic interactions between the bacterial and fungal colonies become evident. Beyond traditional agar plate methods, liquid culture antagonism assays can also be employed as an alternative. In these tests, microbial growth is monitored via optical density measurements, and serial dilutions are subsequently plated to assess both antagonistic interactions and the inoculum population. (Meyers; Furtmann; Jose, 2018; Beal et al., 2020). As an extension of these methodologies, Figure 4 provides a schematic overview of agar plate-based antagonistic assays used to evaluate microbial compatibility in hydrocarbon biodegradation.

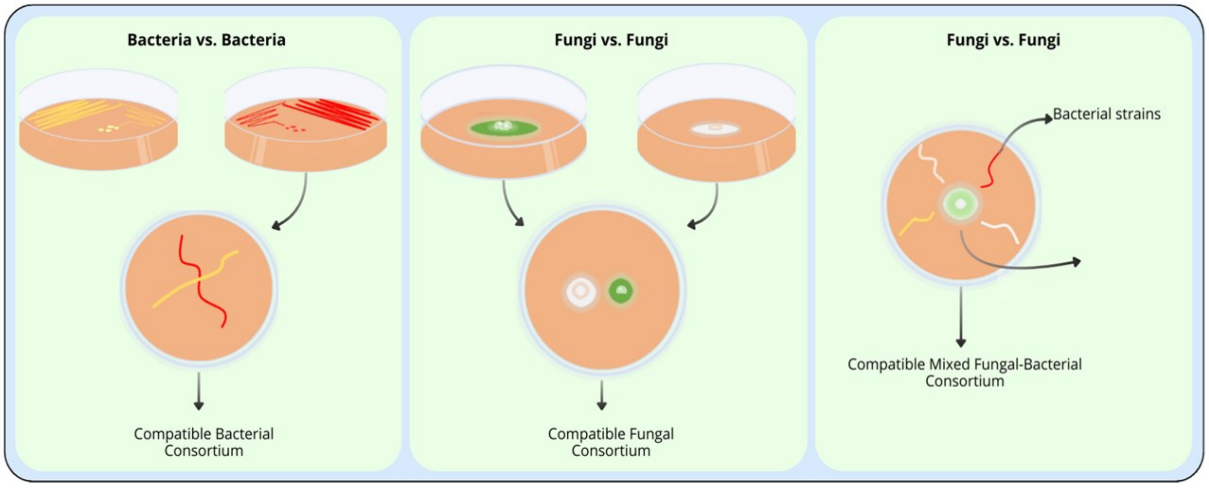

Figure 4. Agar plate-based antagonistic assays for the selection of compatible microbial consortia in hydrocarbon biodegradation.

After selecting species that exhibit no antagonistic interactions, bacterial, fungal, or mixed consortia can be established via co-cultivation in liquid media (Vipotnik; Michelin; Tavares, 2021; Espinosa-Ortiz; Rene; Gerlach, 2022). Alternatively, a pre-inoculation strategy can be employed, wherein individual microbial strains are cultivated separately in liquid medium before the final consortium is assembled by combining the cultures in a 1:1 volumetric ratio to ensure balanced microbial representation (Ebadi et al., 2021; Ramdass; Rampersad, 2023). This approach allows for the optimization of growth conditions for each microbial member, thereby preventing competitive exclusion during the initial establishment phase. As observed in bacterial consortia designed for PAHs biodegradation**,** where pre-inoculated microbial strains exhibited degradation efficiencies surpassing 90% for petroleum hydrocarbons (Behera et al., 2021; Zhou et al., 2023)**.**

For mixed fungal-bacterial cultures, a sequential inoculation strategy described by Ghorbannezhad, Moghimi and Dastgheib (2021) can also be employed, wherein bacterial strains are introduced into the liquid medium five days after the fungal strains have been inoculated**.** In this approach, fungi are initially introduced, followed by the addition of bacteria one week later**.** The results indicated that this sequential inoculation strategy led to 60.76% degradation of pyrene and 73.48% degradation of tetracosane**,** approximately 10% higher than the traditional simultaneous inoculation method**.** The authors attributed this improvement to enhanced initial fungal growth**,** which allowed extracellular fungal enzymes to initiate the breakdown of hydrocarbons, making them more accessible for subsequent bacterial degradation**.** Furthermore, the addition of rhamnolipid significantly enhanced the degradation

of pyrene by approximately 16% and tetracosane by 23%**,** demonstrating the effectiveness of this combined approach**.**

Following the adoption of this screening framework and the formulation of compatible microbial consortia, effectiveness assays can be conducted to evaluate their bioremediation potential. These processes and the underlying mechanisms have been primarily investigated through microcosm studies (Asadirad et al., 2016; Basim et al., 2020; Dehvari et al., 2021; Iyobosa et al., 2021). As simplified artificial ecosystems, microcosms enable the simulation and prediction of natural ecosystem dynamics under controlled conditions (Cao; Li; Li, 2021), thereby providing valuable insights into microbial potential for subsequent applications *in-situ* or *ex-situ* bioremediation strategies.

3.7.3. Immobilization of Microbial Consortia for Enhanced Hydrocarbon Degradation

An alternative strategy to improving bioremediation efficiency involves the use of bio-carrier supports for microbial immobilization, which can enhance biological stability and optimize hydrocarbon degradation processes. These carrier matrices, composed of various materials, serve as protective environments that shield microorganisms from suboptimal environmental conditions and mitigate inhibitory effects arising from competition with indigenous microbial communities (Mrozik; Piotrowska-Seget, 2010). For instance, the immobilization of a bacterial consortium consisting of *Exiguobacterium* sp., *Pseudomonas aeruginosa*, *Alcaligenes* sp., and *Bacillus* sp. in calcium alginate beads supplemented with activated carbon significantly enhanced microbial tolerance to high crude oil concentrations and variable environmental parameters. Compared to its planktonic counterpart, the immobilized consortium demonstrated superior hydrocarbon degradation efficiency, achieving a total petroleum hydrocarbon (TPH) removal rate of 75% (Chen et al., 2017).

Similarly, a microbial consortium composed of *Pseudomonas* sp. PH-1, *Bacillus* sp. PH-2, *Ochrobactrum* sp. PH-3, and *Pseudomonas* sp. PH-4, immobilized in semi-coke carbon spheres, exhibited enhanced biodegradation performance and greater resilience to fluctuating environmental conditions. Specifically, the immobilized consortium degraded 86% of naphthalene and 89% of phenanthrene in a crude oil-contaminated liquid medium, whereas free-floating consortia achieved removal rates of only 30% and 80%, respectively (Shen et al., 2015). A more recent study by Rungsihiranrut et al. (2023) employed an aquaporous gel matrix

to immobilize a bacterial consortium comprising *Sphingobium naphthae* MO2-4 and *Priestia aryabhattai* TL01-2. This research, focused on hydrocarbon degradation in marine environments, demonstrated that the immobilized consortium successfully removed 70% of crude oil in a wave tank system. Furthermore, both strains harbored genes associated with heavy metal resistance and biosurfactant synthesis, underscoring their multifunctional potential for bioremediation applications.

The incorporation of mixed fungal-bacterial consortia into these systems presents another robust approach, leveraging the complementary metabolic capabilities of both groups. A liquid microcosm study using crude oil as the sole carbon source evaluated a mixed microbial consortium consisting of *Acinetobacter* sp. Y2, a biosurfactant-producing bacterium, and *Scedosporium* sp. ZYY, a hydrocarbon-degrading fungus. Both strains were immobilized in sodium alginate and polyvinyl alcohol (PVA) beads. The consortium with a higher bacterial proportion (3:1) exhibited the highest total petroleum hydrocarbon (TPH) removal efficiency, achieving 89.4% degradation. Additionally, this treatment demonstrated superior degradation of medium- and long-chain *n*-alkanes (C11–C20 and C21–C40), with a removal rate of 95%. The increased bacterial proportion was also associated with enhanced biosurfactant production, leading to a 50% reduction in surface tension and a higher emulsification index (E24), suggesting a direct correlation between biosurfactant activity and improved hydrocarbon solubilization. In contrast, treatments with a higher fungal proportion (2:1) exhibited greater efficiency in polycyclic aromatic hydrocarbon (PAH) removal, degrading 70.9%, 69.6%, and 62.3% of 2-, 3-, and 4-ring PAHs, respectively (Atakpa et al., 2023).

These findings highlight the advantages of integrating bacteria and fungi into immobilized consortia, as each microbial group targets different hydrocarbon fractions, resulting in a more comprehensive and efficient degradation process. The use of polymeric matrices such as sodium alginate and PVA not only enhances microbial stability under fluctuating environmental conditions but also facilitates the controlled release of degradative enzymes and biosurfactants. Emphasizing the potential of mixed microbial consortia for both *in situ* and *ex situ* bioremediation strategies, further reinforcing the importance of harnessing microbial diversity and alternative immobilization techniques to optimize petroleum hydrocarbon degradation.

3.8 CONCLUSIONS

This review highlights the multifaceted mechanisms governing hydrocarbon degradation by microbial communities, emphasizing the distinct yet complementary roles of bacteria and fungi. The cooperative interactions between these microorganisms facilitated by biosurfactant production, enzymatic synergy, and metabolic cooperation, significantly enhance the degradation of persistent hydrocarbons, such as polycyclic aromatic hydrocarbons (PAHs), compared to individual isolates. Furthermore, the strategic assembly of bacterial, fungal, or mixed fungal-bacterial consortia presents a highly effective approach for optimizing bioremediation, leveraging synergistic metabolic pathways to accelerate contaminant breakdown and improve mineralization efficiency. In summary, the integration of advanced microbial screening techniques, strategic consortium formulation, and a mechanistic understanding of hydrocarbon degradation provides a robust framework for addressing petroleum pollution. These interdisciplinary approaches not only enhance biodegradation efficiency but also support the development of tailored, eco-friendly solutions for the restoration of contaminated environments.

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4. CHAPTER II

EXPLORING NOVEL FUNGAL-BACTERIAL CONSORTIA FOR ENHANCED PETROLEUM HYDROCARBON DEGRADATION

This chapter corresponds to the published article:

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ABSTRACT

Bioremediation, involving the strategic use of microorganisms, has proven to be a cost-effective alternative for restoring areas impacted by persistent contaminants such as polycyclic aromatic hydrocarbons (PAHs). In this context, the aim of this study was to explore hydrocarbon-degrading microbial consortia by prospecting native species from soils contaminated with blends of diesel and biodiesel (20% biodiesel/80% diesel). After enrichment in a minimal medium containing diesel oil as the sole carbon source and based on 16S rRNA, Calmodulin and β-tubulin gene sequencing, seven fungi and 12 bacteria were identified. The drop collapse test indicated that all fungal and four bacterial strains were capable of producing biosurfactants with a surface tension reduction of ≥20%. Quantitative analysis of extracellular laccase production revealed superior enzyme activity among the bacterial strains, particularly for *Stenotrophomonas maltophilia* P05R11. Following antagonistic testing, four compatible consortia were formulated. The degradation analysis of PAHs and TPH (C5–C40) present in diesel oil revealed a significantly higher degradation capacity for the consortia compared to isolated strains. The best results were observed for a mixed bacterial-fungal consortium, composed of *Trichoderma koningiopsis* P05R2, *Serratia marcescens* P10R19 and *Burkholderia cepacia* P05R9, with a degradation spectrum of ≥91% for all eleven PAHs analyzed, removing 93.61% of total PAHs, and 93.52% of TPH (C5–C40). Furthermore, this study presents the first report of *T. koningiopsis* as a candidate for bioremediation of petroleum hydrocarbons.

Keywords: co-cultures; biodegradation; petroleum hydrocarbons; fungal consortia; bacterial consortia; diesel B20.

4.1 INTRODUCTION

Anthropogenic activities involving the supply chain of petroleum-based products are frequently associated with accidental spills into the environment (Zhang et al., 2018; Ladle et al., 2020). As a result, the exposure to such substances can cause irreversible impacts on the organisms and ecosystems affected (Carmo; Teixeira, 2020). In its composition, crude oil comprises a complex mixture of hydrocarbons (polycyclic aromatic, monoaromatic, aliphatic, and cycloalkanes), asphaltenes, and resins (Schobert, 2013). Among these constituents, polycyclic aromatic hydrocarbons (PAHs) are recognized by their persistence in the environment, attributed to physicochemical properties like low solubility, high molecular weight, and stability resulting from the fusion of two or more aromatic rings (Patel et al., 2020; Barathi et al., 2023). Furthermore, the bioaccumulation capacity of these compounds is associated with cytotoxic, carcinogenic, mutagenic, and teratogenic effects (Mastrangelo; Fadda; Marzia, 1996; Delgado-Saborit; Stark; Harrison, 2011; Mulla et al., 2023). Consequently, the accidental release of these contaminants and their long-term effects are of great concern, emphasizing the need for alternative energy sources as well as innovative

approaches focused on environmental remediation and restoration (Dzionek; Wojcieszyńska; Guzik, 2016; Patel et al., 2020).

The use of biofuels derived from renewable biomass offers an alternative to expanding the global energy matrix (Srivastava et al., 2020). Among the biofuels utilized in the transportation sector, biodiesel oil stands out for its capacity to replace mineral-derived diesel oil or be incorporated into binary blends composed of diesel/biodiesel (Chauhan; Gangopadhyay; Singh, 2009). In Brazil, the production and demand for biodiesel oil have been increasing through public policies, which currently mandate the inclusion of 14% (*v*/*v*) biodiesel in commercial diesel, forming the B14 blend (Kohlhepp, 2010; ANP, 2024). On the other hand, despite biodiesel being described as a biodegradable fuel with low toxicity (Khan; Warith; Luk, 2007; Demirbaş, 2009), mineral diesel oil, which constitutes the largest portion of diesel/biodiesel binary blends, consists of a complex mixture of hydrocarbons known for their harmful effects (Zungum; Imam, 2021), posing a risk in the face of accidental releases associated with the increased usage of these blends.

In response to this environmental challenge, sustainable remediation technologies have been receiving increasing attention (Mishra et al., 2023; Thacharodi et al., 2023). From this perspective, microbial bioremediation has demonstrated significant advantages over physico-chemical techniques that are generally associated with complex pre-treatments, inefficient removal of residual contaminants, and the high overall cost of the process (Fulekar, 2017). This efficient, low-cost biological system can mediate the biotransformation, detoxification, and permanent removal of a wide range of organic contaminants, including high molecular weight PAHs (Dzionek; Wojcieszyńska; Guzik, 2016; Gan et al., 2022). However, in order to establish a system that promotes the consumption and mineralization of contaminants, the selection of microorganisms equipped with appropriate metabolic mechanisms is essential (Fritsche; Hofrichter, 2008).

As a source for bioprospecting, environments impacted by petroleum hydrocarbons have shown a strong influence on the modulation of native microbial communities (Suja et al., 2014; Wu et al., 2017). These findings are supported by studies revealing numerous hydrocarbon-degrading fungal genera in contaminated areas, such as *Aspergillus*, *Penicillium*, *Pleurotus*, *Trametes*, *Fusarium,* and *Trichoderma* (Bovio et al., 2017; Barnes et al., 2018; Benguenab; Chibani, 2020; Medaura et al., 2021), along with some bacteria like *Alcanivorax*, *Pseudomonas*, *Bacillus*, *Rhodococcus*, *Sphingomonas*, *Burkholderia*, *Paraburkholderia*,

Serratia, and *Stenotrophomonas* (Siles; Margesin, 2018; Yuniati, 2018; Akhtar; Mannan, 2020). Among the members of this microbial community, several fungal species are recognized for their secretion of important oxidative enzymes, such as laccases (LaC, EC:1.10. 3.2), manganese peroxidases (MnP, EC:1.11.1.13), lignin peroxidases (LiP, EC:1.11.1), and versatile peroxidases (VP, EC:1.11.1.16) (Kadri et al., 2017). These enzymes act nonspecifically, catalyzing the simultaneous oxidation of a large number of aromatic and non-phenolic compounds, with the potential to degrade different types of hydrocarbons (Giardina et al., 2010; Thacharodi et al., 2023). For example, the ability of a *Penicillium oxalicum* strain to produce LaC, MnP, and VP has demonstrated a direct correlation with increased degradation of high molecular weight PAHs, including anthracene, dibenzothiophene, phenanthrene, and dibenzofuran (Aranda et al., 2017). Similarly, Pozdnyakova et al. (2018) reported that the combined activity of LaC and VP expressed by a *Pleurotus ostreatus* strain was responsible for converting phenanthrene and anthracene into quinones that integrated into the basal metabolism, leading to the mineralization of these compounds.

Beyond these fungal mechanisms, numerous bacterial species are recognized for their significant role in PAH degradation, employing mixed-function oxygenase enzymes, such as monooxygenases, dioxygenases, peroxidases, hydroxylases, and dehydrogenases, to efficiently metabolize these compounds (Ghosal et al., 2016). For example, the combined activity of enzymes like alkane hydroxylase, alcohol dehydrogenase, and laccases, produced by *Pseudomonas stutzeri* NA3 and *Acinetobacter baumannii* MN3, have been shown to enhance crude oil degradation, achieving removal efficiencies of 84% and 78%, respectively (Parthipan et al., 2017). In another study, a functional analysis of *Delftia* sp. and *Burkholderia* sp. during phenanthrene degradation revealed the 'phn' genomic island as an integrative element containing key genes for PAH degradation, including phenanthrene dioxygenase, which catalyzes the initial oxidation of the aromatic ring. The presence of hydrocarbons in the medium upregulated *phn* genes, directly linking their expression to PAHs exposure. Additionally, proteomic profiling revealed increased expression of proteins like *PhnF* and *PhnJ*, along with downstream catabolic enzymes, underscoring these bacteria's adaptive mechanisms in PAH-contaminated environments (Hickey; Chen; Zhao, 2012).

Another important metabolic strategy known to enhance the degradation of hydrocarbons is the production of biosurfactants by certain species of fungi and bacteria (Da Silva et al., 2021; Imam et al., 2022). These active surface molecules reduce the interfacial and surface tension, increasing the contact surface area of the hydrophobic portions of

contaminants, which leads to enhanced bioavailability for subsequent biodegradation (Zhou et al., 2011; Ławniczak et al., 2020). According to Mnif et al. (2014), a proportional relationship between the production of biosurfactants by a *Bacillus subtilis* strain and the biodegradation of diesel oil and kerosene seems to be the rule of thumb. Combined with these mechanisms, mutualistic associations between bacteria and fungi have demonstrated a critical role in the biodegradation of complex mixtures of hydrocarbons (Patowary et al., 2016).

Since a single microorganism typically metabolizes only a limited range of hydrocarbons, synergism through complementary biochemical pathways enables one organism to degrade a metabolite produced by another, in order to complete the hydrocarbon degradation process or alleviate potential toxic/inhibitory effects (Kamyabi; Nouri; Moghimi, 2017; Ghorbannezhad; Moghimi; Dastgheib, 2018).

In this context, bioremediation through the use of mixed cultures of fungi and bacteria equipped with different metabolic mechanisms can enhance the extent and efficacy of biodegradation (El Hanafy et al., 2016; Ghorbannezhad; Moghimi; Dastgheib, 2021). Atapka et al. (2022), for example, suggest that combining fungal strains with biosurfactant-producing bacteria effectively improves the degradation of petroleum hydrocarbons. According to the authors, the use of a consortium composed by *Acinetobacter* sp. and *Scedosporium* sp. provided higher rates of total petroleum hydrocarbon (TPH) degradation than when the strains were analyzed individually. In another study, Ameen et al. (2016) demonstrated that a consortium composed of *Alternaria alternata*, *Aspergillus terreus*, *Cladosporium sphaerospermum*, *Eupenicillium hirayamae*, and *Paecilomyces variotii*, isolated from hydrocarbon-contaminated mangrove sediments, was able to completely degrade the main hydrocarbons present in diesel oil. As observed, the synergistic effect of the combined genotypes for the production of complex enzymes (Lac, LiP, and MnP) was significantly higher compared to the strains evaluated alone.

Aligned with this information, the primary objective of this study was to develop microbial consortia with potential for application in the bioremediation of petroleum hydrocarbons. To achieve this goal, fungal and bacterial strains were isolated from experimental areas intentionally contaminated with B20 biodiesel (20% *v*/*v* biodiesel and 80% *v*/*v* diesel). After identifying the isolates, different combinations of microbial consortia were designed based on the results for the screening of extracellular laccases, biosurfactants, and species compatibility. These novel consortia and individual strains, including the *Trichoderma koningiopsis* strain evaluated in this context for the first time, were subsequently tested for their

diesel oil degradation potential. The results highlighted the effectiveness of these microbial partnerships in targeting and degrading complex hydrocarbon pollutants, suggesting promising applications in environmental bioremediation.

4.2. MATERIALS AND METHODS

4.2.1. Collecting Area

The samples utilized in the present study were collected at the Ressacada Experimental Farm, in Florianópolis, southern Brazil (latitude 27°30′ S, longitude 48°30′ W), where controlled contamination procedures with fuels and biofuels have been monitored for 25 years (1998–2023) through research projects linked to PETROBRAS in partnership with the Ressacada Environmental Research Center (REMA) and the Federal University of Santa Catarina (UFSC). Based on historical analytical data, the experimental release areas P05 (B100-ANM) and P10 (B20-BAF) were selected to prospect petroleum hydrocarbon-degrading microorganisms and analyze the current concentration of contaminating hydrocarbons. The experimental area P05 (B100-ANM) was contaminated with 100 L of B20 biodiesel (20% *v*/*v* soybean biodiesel and 80% *v*/*v* diesel), which had been subjected to natural attenuation remediation starting in 2008. Area P10, contaminated with 100 L of B20 biodiesel (20% *v*/*v* palm biodiesel and 80% *v*/*v* diesel), underwent remediation through bioaugmentation combined with Fe_2O_3 in 2017 (Müller et al., 2017).

Soil samples from the selected areas were collected on April 28, 2021, using a Dutch auger. With the aid of the equipment, three perforations were made at points near the contamination source well. According to previous data on the depth of contaminant release in the soil, samples were collected at 1.5 m depth for area P05 and 1.8 m depth for area P10. After collection, mixed soil compositions were prepared by homogenizing the samples. Approximately 500 g of soil kept at a temperature of 4 °C were sent to the laboratory for analysis.

4.2.2. Evaluation of PAHs and BTEX Levels at Sampling Sites

Employing the EPA 3550C method with some modifications, approximately 4 g of dry, homogenized soil was transferred to 20 mL vials for the extraction of PAHs (USEPA, 2007). Dichloromethane (CH_2Cl_2) was chosen as the solvent for ultrasonic extraction and the process

was performed under a Cole-Parmer 8890E-MTH ultrasonic device at a frequency of 55 Hz for 30 min. This process was repeated three times, using 6 mL of solvent per step. After extraction, the organic phase extracts were allowed to settle for 10 min to facilitate phase separation. The extracts were then vacuum filtered using PTFE membranes with a pore size ≤ 0.45 μm. The final extract was concentrated under a gentle stream of nitrogen gas and transferred to vials. PAH concentrations were evaluated using an HP 6890 Series II gas chromatograph with a Headspace auto-sampler G1888, a flame ionization detector (FID), and an HP5 capillary column (J&W Scientific, Agilent Technologies, Santa Clara, USA). Helium served as the carrier gas at a flow rate of 3.0 $mL \cdot min^{-1}$. The injector and detector temperatures were set at 260 °C and 320 °C, respectively. The oven temperature was programmed as follows: an initial ramp from 60 °C to 200 °C at a rate of 4 °C min^{-1}, followed by a final ramp from 200 °C to 280 °C at a rate of 2 °C min^{-1}.

For the extraction of BTEX (benzene, toluene, ethylbenzene, and xylenes), approximately 1 g of soil was weighed into 20 mL vials, and the volume was adjusted to 10 mL with ultrapure water, following the EPA 5021A method combined with EPA 8015D (USEPA, 1996, 2003). Quantification was performed using a gas chromatograph with an HP1 column. Helium was again used as the carrier gas at a flow rate of 3.0 $mL \cdot min^{-1}$. The injector and detector temperature were set at 300 °C and the oven temperature was ramped from 70 °C to 120 °C at a rate of 6 $°C \cdot min^{-1}$. The detection limits for each individual compound are detailed in other sources (Corseuil et al., 2011; Ramos et al., 2014).

4.2.3. Enrichment and Isolation of Hydrocarbon-degrading Strains

The isolation and selection of fungal and bacterial species with potential for biodegrading petroleum hydrocarbons were conducted using the enrichment technique in minimal medium with some modifications (Poddar; Sarkar; Sarkar, 2019). Initially, 5 g of soil samples from the contaminated areas were transferred to 250 mL Erlenmeyer flasks containing 80 mL of Bushnell Haas—BH medium—composed of (g/L): 1 g K_2HPO_4, 0.2 g $MgSO_4$, 1 g KH_2PO_4, 0.02 g $CaCl_2$, 1 g NH_4NO_3, 0.05 g FeCl2 (Bushnell; Haas, 1941), enriched with 1% (*v*/*v*) filtered commercial diesel oil as the only carbon source, which was added after sterilization. The flasks were maintained on an orbital shaker (150 rpm, 28 °C), with samples collected at the following two distinct periods: at 7 and 14 days after incubation. The enriched cultures were then serially diluted (10^{-6} to 10^{-9}) and spread over sterile plates with Nutrient

Agar - NA (BD Difco, Franklin Lakes, USA), and Malt Extract Agar - MEA (Millipore, Burlington, USA), and incubated at 28 °C ± 2 °C for 7 days. Resistive colonies, selected based on visual differences in morphology, size, and coloration, were re-streaked to obtain pure colonies. The axenic cultures were then subjected to a second enrichment procedure in BH mineral medium enriched with 5% (*v*/*v*) diesel oil. After the incubation period, bacterial isolates that still exhibited viable colonies were further assessed for cell wall type, morphology, and arrangement using Gram staining (Adams, 1975). Micromorphology of fungal isolates was assessed through the microculture technique (García De Lomas et al., 1981). To observe additional taxonomic features, such as colony color, texture, production of soluble pigments and exudates, the fungal isolates were point-inoculated onto sterile plates containing Czapek Yeast Extract Agar—CYA (Pitt, 1979)—Yeast Extract Sucrose Agar—YES (Frisvad, 1981)—and MEA. The plates were then incubated at 28 °C for 7 days.

4.2.4. Identification of Hydrocarbon-Degrading Fungi and Bacteria

Total genomic DNA from the isolated fungi and bacteria was extracted using the Plant/Fungi DNA Isolation Kit (Norgen Biotek, Thorold, Canada) and the DNeasy Kit (Qiagen, Hilden, Germany), respectively. DNA concentration and purity were assessed using a NanoVue Plus spectrophotometer (GE). For fungal species identification, the genomic DNA was subjected to amplification of partial regions of the β-tubulin gene (BenA) using the primers Bt2a (5′-GGT AAC CAA ATC GGT GCT GCT TTC-3′) and Bt2b (5′-ACC CTC AGT GTA GTG ACC CTT GGC-3′) (Glass; Donaldson, 1995). Additionally, partial regions encoding the Calmodulin (CaM) gene were amplified using primers CMD5 (5′-CCG AGT ACA AGG AGG CCT TC-3′) and CMD6 (5′-CCG ATA GAG GTC ATA ACG TGG-3′) (Hong et al., 2006). These genes were selected for their ability to provide higher resolution in distinguishing closely related fungal species, which is particularly useful for species within the *Aspergillus* and *Penicillium* genera (Visagie et al., 2014).

Genomic DNA extracted from isolated bacteria was amplified using primers 27F (-AGA GTT TGA TCM TGG CTC AG-) (Lane, 1991) and 1492R (-GGT TAC CTT GTT ACG ACT T) (Turner et al., 1999) to obtain 16S rRNA gene fragments. PCR products were visualized on a 1% agarose gel stained with GelRed (Invitrogen, Carlsbad, USA). The amplicons were purified using the PCR Products Purification Kit (Ludwig, Alvorada, Brazil) and sent for

Sanger sequencing at ACTGene Company (Brazil). The sequences were submitted to the GenBank database to align with published sequences using NCBI BLASTN. The nucleotide sequences of isolates were deposited in GenBank and accession numbers were obtained.

4.2.5. Phylogenetic Analysis

A Kimura 2-parameter phylogenetic tree (K2 + G) was constructed with the MEGA 7 software version 7.0 (Kumar; Stecher; Tamura, 2016), based on the maximum likelihood (ML) test for fungal sequences and the Neighbor-Joining (NJ) test for bacterial sequences, with 1000 bootstraps (Felsenstein, 1981; Saitou; Nei, 1987). In addition to 16S rRNA gene amplification, bacterial isolates were further differentiated by comparing the repetitive DNA banding profiles generated using the BOX-PCR technique (Versalovic et al., 1994). Genomic DNA was used as the template for amplification, following the procedure described by Versalovic et al. **(**1994**)** and using the primer A1R (5′-CTA CGG CAA GGC GAC GCT GAC G-3′). A control reaction was performed by substituting genomic DNA with ultrapure water. After the amplification reaction, the products were visualized through electrophoresis on a 1.2% (*w*/*v*) agarose gel, run at a voltage of 3 volts per centimeter. The gel image was photographed using a UV transilluminator and the ChemiDoc MP Digital Imaging System (Bio-Rad, Hercules, USA). Bands were clustered based on similarity, and a dendrogram was constructed using the UPGMA method and Jaccard coefficient in software NTSYS version 2.1 (Sokal, 1958; Rohlf, 2000).

4.2.6. Screening for Biosurfactants

To evaluate the potential production of biosurfactants, twelve bacterial strains were initially cultivated for 48 h (130 rpm, 30 °C) in 250 mL Erlenmeyer flasks containing 50 mL of nutrient broth (BD Difco). Subsequently, 1% *v*/*v* of the inoculum was transferred to Erlenmeyer flasks containing 50 mL of Mineral Salts Medium—MSM—which consists of (g/L): 7.6 g Na_2HPO_4, 4.43 g KH_2PO_4, 6 g $(NH_4)_2SO_4$, 0.4 g $MgSO_4$, 0.4 g $CaCl_2$, and 2 mL of a trace element solution (20.1 g disodium EDTA, 16 g $FeCl_3 \cdot 6H_2O$, 0.18 g $CoCl_2 \cdot 6H_2O$, 0.18 g $ZnSO_4 \cdot 7H_2O$, and 0.16 g $CuSO_4 \cdot 5H_2O$) (Ray et al., 2021). The medium was supplemented with 3% (*v*/*v*) filtered soybean oil, which was added after sterilization. The flasks were incubated in an orbital shaker (130 rpm, 30 °C) for 7 days. After incubation, the total volume of each culture

was transferred to 50 mL Falcon tubes and centrifuged at 4.500 rpm for 15 min. The supernatant was then used for subsequent tests.

The fungal species were initially cultivated on MEA for 4 to 7 days at 28 °C. Two mycelial disks (5 mm) were then transferred to 250 mL Erlenmeyer flasks containing 50 mL of medium composed of (g/L): 3 g KH_2PO_4, 5 g $NaNO_3$, 3 g $MgSO_4 \cdot 7H_2O$, 3 g $FeSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot H_2O$, 0.01 g $ZnSO_4$, and 0.2% (*w*/*v*) yeast extract (Othman et al., 2022). The medium was supplemented with 3% (*v*/*v*) soybean oil, added after sterilization. The cultures were incubated on an orbital shaker (120 rpm, 28 °C) for 7 days. After the incubation period, the total volume of each culture was transferred to 50 mL Falcon tubes and were subjected to ultrasonic baths at room temperature for 5 min, to release biosurfactants adhered to the biomass and cell membrane. The samples were then centrifuged at 4.500 rpm for 15 min, and the supernatant was used to evaluate biosurfactant production via the pendent-drop method (Lecomte Du Noüy, 1925; Ebnesajjad, 2010). The pendent-drop assay is based on the destabilization of liquid oil droplets by surfactants. The surface tension at the water–oil interface causes an oil droplet to be repelled by a hydrophilic surface. However, when the liquid on the surface contains surfactants, they reduce the surface tension, causing the droplet to spread or collapse (Chandankere et al., 2014; Da Silva et al., 2021). The biosurfactant production cultures were prepared in triplicate, with non-inoculated flasks serving as controls.

The determination of surface tension was conducted using approximately 3 mL of culture supernatant. Surface tension measurements (in $mN \cdot m^{-1}$) were performed using a goniometer 250-F1 (Ramé-Hart Instrument Co., Succasunna, USA) (Supplementary Figure S1). The goniometer was calibrated using distilled water, and the syringe used was rinsed three times with distilled water and twice with the sample to be analyzed. The DROPimage Advanced software was employed to analyze the curvature profile of the pendant drop formed at the tip of the needle. Ten measurements were taken at one second intervals for each reading, and the final result was the average of these ten measurements. All measurements were performed in triplicate at room temperature (25 °C to 28 °C). Furthermore, the percentage of surface tension reduction (STR) was calculated to assess the potential production of biosurfactants. The calculation was derived from the ratio between the negative variation in surface tension during cultivation and the surface tension of the negative control, multiplied by 100 (Da Silva et al., 2021).

4.2.7. Screening for Laccases

In a preliminary screening step, all isolated fungal and bacterial strains were assessed for qualitative laccase activity to identify those with the potential for further quantitative analysis. Following the methodology adapted from Karp et al. (2012), mycelial disks from seven isolated fungal strains were used as inoculum. Two disks were transferred to 250 mL Erlenmeyer flasks containing 80 mL of modified Czapek Yeast Extract broth (g/L): 10 mL of concentrated Czapek solution [30 g $NaNO_3$, 5 g KCl, 5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g $ZnSO_4 \cdot 7H_2O$, and 100 mL of distilled water], 1 g K_2HPO_4, 2.5 g yeast extract, and 15 g dextrose. The medium was supplemented with 150 µM $CuSO_4 \cdot 5H_2O$, which was pre-filtered and added after sterilization. The flasks were incubated on an orbital shaker (120 rpm, 28 °C) for 12 days, with 1 mL aliquots collected every 48 h.

For bacterial species, a pre-culture in nutrient broth was prepared for the twelve selected strains. An aliquot of 1% *v*/*v* of the pre-culture was transferred to 250 mL Erlenmeyer flasks containing 50 mL of modified MSM broth containing the following (g/L): 2.5 g yeast extract, 1.2 g dextrose, 7 g NaCl, 4 g KH_2PO_4, 8 g K_2HPO_4, and 0.18 g $MgSO_4$, pH 7 (Rajeswari M; Vennila K; Bhuvaneswari V, 2015). The medium was supplemented with 500 µM $CuSO_4 \cdot 5H_2O$, pre-filtered, and added after sterilization. The flasks were incubated on an orbital shaker (130 rpm, 30 °C) for 96 h, with 1 mL aliquots collected every 24 h.

The presence of extracellular laccases was assessed using the substrate ABTS-2,2′-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid] (Merck, Rahway, USA). The enzymatic reaction was prepared for a final volume of 1 mL, composed of the following: 800 µL of 100 mM sodium acetate buffer (pH 4.5), 100 µL of 20 mM ABTS (in distilled water), and 100 µL of crude enzyme extract obtained by centrifuging the collected aliquots at 13.000 rpm for 5 min. The tubes were mixed by inversion and incubated in a dry bath EchoTherm (Torrey Pines Scientific, Carlsbad, USA) for 20 min at 40 °C. After incubation, microtubes (1.5 mL) exhibiting enzymatic activity showed the development of a green–bluish coloration, indicating substrate oxidation. A control reaction was performed by replacing the crude enzyme extract with 100 µL of the control culture medium. All assays were conducted in triplicate.

4.2.8. Growth conditions for Quantitative Evaluation of Laccases

The species demonstrating the highest levels of oxidation in microtube assays were subsequently monitored under submerged cultivation to determine the levels of extracellular laccase activity. For the selected fungal species, cultivation was performed in 250 mL Erlenmeyer flasks containing 100 mL of modified Czapek Yeast Extract broth, as previously described. Once inoculated, the cultures were maintained on an orbital shaker (110 rpm, 28 °C) for 14 days, with samples collected every 24 h to determine extracellular laccase activity. Bacterial cultivation was carried out in 250 mL Erlenmeyer flasks containing 80 mL of the modified MSM broth. The flasks, inoculated with 1% *v*/*v* of a pre-inoculum, were incubated on an orbital shaker (130 rpm, 30 °C) for 6 days, with samples collected every 24 h to determine extracellular laccase activity and microbial growth by measuring the optical density (OD) of the cultures at 600 nm using a spectrophotometer DR3900 (HACH, Loveland, USA).

4.2.9. Measurement of Extracellular Laccase Activity

The activity of extracellular laccases was assessed by monitoring the oxidation of the ABTS substrate. The reaction mixture consisted of 800 µL of 50 mM sodium acetate buffer (pH 4.5), 100 µL of 10 mM ABTS (in distilled water), and 100 µL of crude enzyme extract obtained by centrifuging the collected aliquots at 13.000 rpm for 5 minutes (Hou et al., 2004). Formation of the $ABTS^{+}$ radical cation was tracked by measuring an absorbance increase at 420nm using a spectrophotometer. Readings were taken every minute over a 5-minute period at room temperature. The control sample consisted of the respective culture media without inoculum. One unit (U) of laccase activity was defined as the amount of enzyme that oxidizes 1 µmol of ABTS per minute at 25 ºC, using a molar extinction coefficient for the $ABTS^{+}$ radical cation ($\varepsilon 420nm = 36{,}000\ M^{-1}\ cm^{-1}$) (Johannes; Majcherczyk, 2000). All assays were conducted in triplicate. Laccase activity was determined based on the formula described by Leonowicz and Grzywnowicz (1981), and expressed in U/mL:

$$(\mathrm{U/mL}) = \frac{\Delta\mathrm{Abs} \times \mathrm{V} \times 10^{6}}{\varepsilon \times \mathrm{Ve} \times \Delta\mathrm{t}}$$

Where: ΔAbs represents the difference in absorbance values obtained; V is the total sample volume (mL); 10^{6} is the factor used to convert moles to micromoles (µmoles); ε is the molar extinction coefficient for the $ABTS^{+}$ radical cation; Ve is the volume (mL) of crude enzyme extract used; and Δt is the reaction time (min).

4.2.10. Selection and Assembly of Microbial Consortia

Considering the results of the biosurfactant and laccase assays, six bacterial strains (P05R8; P05R9; P05R11; P05R12; P10R13; P10R19) and five fungal strains (P05R1; R05R2; P05R3; P10R5; P10R7) were selected for analysis of growth dynamics when co-cultivated. This step aimed to formulate microbial consortia capable of degrading petroleum hydrocarbons.

To ensure the stability of the formed consortia, potential antagonistic behavior among the different strains was evaluated by confrontation on sterile plates containing Toyama's medium composed by the following (g/L): 1 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 3 g KH_2PO_4, 0.5 g NaCl, 0.001 g $FeSO_4$, and 10 g dextrose (Wunder et al., 1994), supplemented with 1% *v*/*v* diesel oil, added after autoclaving. Microbial confrontations occurred in the following order: (fungus vs. fungus; bacteria vs. bacteria; bacteria vs. fungus). For the fungus vs. fungus antagonism assay, two species per plate were point-inoculated approximately 2 cm apart. The plates were incubated at 28 °C until inhibition halos or antagonistic effects between fungal colonies were observed. Antagonism between bacteria was evaluated by inoculating two strains per plate following a grid pattern, allowing direct contact between the strains. The plates were incubated at 30 °C until antagonistic effects between the colonies were observed. In the bacteria vs. fungus antagonism assay, fungi were inoculated individually at the center of the plates and then incubated at 28 °C for 4 days. After partial development of the fungal colonies, six bacterial strains were transferred to the periphery of the plates, streaking towards the center of the fungal colonies. The plates were re-incubated at 28 °C until antagonistic effects between the colonies were observed (Zafra et al., 2017). Based on the compatibility of the strains, consortia of mixed cultures of bacteria, fungi, and bacteria-fungi were formulated.

4.2.11. Biodegradation of Diesel Oil by Individual Strains and Microbial Consortia

The ability to biodegrade diesel oil and its hydrocarbons (PAHs and TPH) was evaluated in 250 mL Erlenmeyer flasks containing 80 mL of BH medium and 1% (*v*/*v*) diesel oil (Ghorbannezhad; Moghimi; Dastgheib, 2018). Both mixed and individual strains were assessed, including the mixed bacteria–fungi consortium FFB1 (P05R2, P05R3, and P10R19), bacterial consortium BB1 (P05R11 and P10R19), fungal consortium FF1 (P05R2 and P05R3), mixed bacteria–fungi consortium FBB1 (P05R2, P10R19, and P05R9), individual fungal strain

P05R2, individual fungal strain P05R3, individual bacterial strain P10R19, and individual bacterial strain P05R9.

Initially, for the formulation of the consortia, fungi and bacteria were cultivated individually. Bacterial strains were inoculated into 250 mL Erlenmeyer flasks containing 80 mL of nutrient broth. The flasks were incubated in an orbital shaker (120 rpm, 28 °C) for 24 h. Subsequently, the cells were harvested by centrifugation at 12.000 rpm for 5 min, washed twice in sterile 0.9% NaCl solution, and resuspended in liquid BH medium (Ganesh; Lin, 2009). The cell suspension concentration was adjusted to an optical density of 1 at 600 nm using a spectrophotometer. Cell density was estimated by plate count technique on NA agar, using serial dilutions (10^{-3} to 10^{-7}). Finally, bacterial consortia were prepared by combining the cultures in a 1:1 volumetric ratio (Ebadi et al., 2021). The prepared consortia and the previously mentioned individual bacterial cultures were inoculated at a 1.2% ratio into the flasks containing BH medium supplemented with diesel (Atakpa et al., 2022). Fungal strains were individually inoculated into plates containing MEA and incubated for 7 days at 28 °C. Subsequently, consortia were prepared using two mycelial disks (5 mm) per strain, taken from the edges of the colonies (Benguenab; Chibani, 2020). The disks of individual fungal strains and fungal consortia were transferred to the flasks containing BH medium supplemented with diesel. For flasks containing mixed fungi–bacteria consortia (FFB1 and FBB1), the sequential inoculation method described by Ghorbannezhad, Moghimi, and Dastgheib (2018) was adopted, where bacterial strains were added to the BH liquid medium five days after the addition of the fungal strains. The flasks were incubated in an orbital shaker (130 rpm, 28 °C) for 15 days. Abiotic control experiments were prepared by incubating diesel oil in BH medium without inoculum. All assays were performed in triplicate. After the incubation period, samples were collected to assess the viability of the consortia and to determine potential antagonistic interactions. Aliquots were serially diluted (10^{-6} to 10^{-9}) and spread onto sterile plates with NA and MEA and incubated at 28 °C ± 2 °C for 7 days. Subsequently, the cell biomass was removed from the culture medium by centrifugation at 7000 rpm for 15 min. The remaining hydrocarbons in the supernatant were extracted using solid phase (SPE) cartridges, according to the EPA 525.2 method (Eicheelberger; Behymer; Budde, 1995), and measured by gas chromatography. The efficacy of the consortia and individual strains was expressed as a percentage of degradation (%) for the hydrocarbons (PAHs and TPH). This was calculated based on the difference between the average concentrations in the control samples and the average concentrations in the inoculated samples, multiplied by 100.

4.2.12. Statistical Analyses

Statistically significant differences among the experimental treatments were analyzed using analysis of variance (ANOVA) followed by the Scott–Knott multiple comparison test at a 0.05 significance level. Homogeneity of variance was assessed using Bartlett's test, and normality of residuals was verified using the Shapiro–Wilk test (Jelihovschi; Faria; Allaman, 2014; R Studio Team, 2021).

4.3. RESULTS

4.3.1. Evaluation of PAHs and BTEX Levels at Sampling Sites

Surprisingly, even a decade after the release of these contaminants in area P05 (2008), the current study revealed residual concentrations of numerous PAHs and significantly elevated levels of BTEX (Table 1). For more precise comparison parameters, we utilized the definitions provided by the National Environment Council (CONAMA, Brazil), which, through Resolution No. 420/2009, establishes guideline values for soil contamination prevention and sets concentration limits for these substances. The analysis of samples from this area indicated that monoaromatic hydrocarbons such as benzene, toluene, and xylenes had concentrations exceeding the recommended limits for soil. Among the PAHs, only naphthalene and benzo(a)anthracene were detected in concentrations above the established limits. However, it is important to note that reference values for some types of PAHs are not covered by the regulatory agency's guidelines, as presented in Table 1. In area P10, contaminated in 2017, concentrations above the recommended average were detected for xylenes, toluene, naphthalene, and anthracene.

Table 1. Concentration of hydrocarbons at sampling sites.

Hydrocarbons (mg/g)*	Area [P05]	Area [P10]	Limit (CONAMA)**
Benzene	8.35×10^{-5}	0	3.00×10^{-5}
Toluene	5.17×10^{-4}	1.92×10^{-6}	1.40×10^{-4}
Ethylbenzene	2.77×10^{-6}	4.40×10^{-4}	6.20×10^{-6}
p-xylene	5.50×10^{-6}	1.70×10^{-6}	1.30×10^{-4}
o-xylene	2.94×10^{-6}	5.20×10^{-4}	1.30×10^{-4}

Table 1. Continuation

Hydrocarbons (mg/g)*	Area [P05]	Area [P10]	Limit (CONAMA)**
Naphthalene	7.49×10^{-4}	6.02×10^{-4}	1.20×10^{-4}
Methyl-naphthalene	5.38×10^{-6}	1.56×10^{-2}	Ni
Acenaphthylene	3.85×10^{-4}	5.59×10^{-4}	Ni
Acenaphthene	8.21×10^{-6}	7.75×10^{-6}	Ni
Fluorene	2.97×10^{-5}	2.78×10^{-4}	Ni
Phenanthrene	5.67×10^{-6}	1.44×10^{-4}	3.30×10^{-6}
Anthracene	1.91×10^{-5}	3.90×10^{-4}	3.90×10^{-5}
Fluoranthene	6.64×10^{-5}	3.98×10^{-4}	Ni
Pyrene	6.96×10^{-4}	7.66×10^{-4}	Ni
Benzo(a)anthracene	6.89×10^{-4}	3.28×10^{-5}	2.50×10^{-5}
Chrysene	5.59×10^{-5}	2.95×10^{-4}	8.10×10^{-6}
Benzo(b)fluoranthene	0	1.07×10^{-4}	Ni
Benzo(a)pyrene	2.31×10^{-6}	3.92×10^{-5}	8.70×10^{-4}
Dibenzo(a,h)anthracene	1.37×10^{-6}	1.79×10^{-5}	8.0×10^{-5}
Benzo(g,h,i)perylene	5.62×10^{-6}	6.99×10^{-6}	5.70×10^{-4}
Indeno(1,2,3-CD)pyrene	1.21×10^{-5}	3.34×10^{-6}	3.10×10^{-5}

*Gas chromatography results expressed in mg of hydrocarbon per gram of soil; **Limit concentrations established by CONAMA; (Ni) not reported.

4.3.2. Enrichment and isolation of hydrocarbon-degrading microbial strains

A total of 19 isolates exhibited viable colonies after two enrichment stages in minimal mineral medium containing commercial diesel oil as the sole carbon source. Of these isolates, 8 originated from area P05, while 11 were from area P10. They were designated using the following nomenclature: P (05 or 10) + R (REMA) + Nº (from 1 to 19). Macro- and micromorphological analyses initially segregated them into two distinct groups, comprising 7 fungi and 12 bacteria. Among the fungal isolates, contrasting morphotypes were observed in MEA, CYA, and YES culture media (Supplementary Figure S2). Isolates P05R1, P05R3, P10R4, P10R5, P10R6, and P10R7 exhibited velvety textured colonies with radial fissures, grooves on the reverse side, and colors ranging from white, cream, orange, to predominantly green. The presence of septate hyphae with conidiophores (either monoverticillate or biverticillate) and short phialides supporting chains of conidia indicated that these isolates were consistent with the genus *Penicillium* (Visagie et al., 2014), likely representing multiple species and/or strains of the genus. In contrast, isolate P05R2 maintained consistent characteristics across different media, presenting white filamentous cotton-like colonies with rapidly

developing aerial mycelium (2 to 4 days), unlike the first group of fungi (7 to 10 days depending on the medium). Micromorphological analyses identified the presence of septate hyphae and smooth globose conidia. Based on the information provided by the study of Samuels et al. (2006), the isolate exhibited similarities with species of the genus *Trichoderma.* Regarding the 12 bacterial isolates, colonies ranged from white to yellow with various textures (opaque, shiny, translucent, and gelatinous). Approximately 25% of the isolates were Gram-positive, while 75% stained as Gram-negative.

4.3.3. Identification of Hydrocarbon-Degrading Fungi and Bacteria

The isolated fungi belonged to the phylum *Ascomycota*, confirming the results of macro- and micromorphological analyses. Comparison of partial sequences of the β-tubulin and Calmodulin genes with those available in the NCBI GenBank revealed six species in the genus *Penicillium* and one species in the genus *Trichoderma* (Table 2).

Table 2. Fungal species identified through sequencing of partial regions of the β-tubulin and Calmodulin genes.

Strain	Closest Match at NCBI (%)		Access number (GenBank)	
	BenA	*CaM*	*BenA*	*CaM*
P05R1	*Penicillium janthinellum* (99.80%)	*Penicillium janthinellum* (100%)	PP942382	PP942390
P05R2	*Trichoderma koningiopsis* (99.12%)	*Trichoderma koningiopsis* (95.88%)	PP942383	PP988131
P05R3	*Penicillium janthinellum* (98.07%)	*Penicillium janthinellum* (97.18%)	PP942384	PP942391
P10R4	*Penicillium pulvillorum* (99.19%)	*Penicillium pulvillorum* (98.72%)	PP942385	PP942392
P10R5	*Penicillium simplicissimum* (97.28%)	*Penicillium* sp. (96.82%)	PP942386	PP942393
P10R6	*Penicillium pulvillorum* (98.98%)	*Penicillium pulvillorum* (99.09%)	PP942387	PP942394
P10R7	*Penicillium pulvillorum* (99%)	*Penicillium pulvillorum* (98.57%)	PP942388	PP942395

According to the collecting areas, some distinct species were identified in area P10 (*P. janthinellum* and *T. koningiopsis*) and P05 (*Penicillium* sp. and *Penicillium pulvillorum*). The phylogenetic tree, constructed from the alignment of *BenA* and *CaM* gene sequences for the reference strains of the *Penicillium* section *Lanata-Divaricata* showed the grouping of strains into clades of their respective type species (Supplementary Figures S3 and S4). In contrast, the *BenA* gene sequence survey for strain P05R2, previously identified as *T. koningiopsis*, returned

limited data on type species, making phylogenetic tree construction unfeasible. However, partial sequences of the *CaM* gene, described as a secondary marker, have proven useful in the phylogenetic resolution of species in the genus *Trichoderma*, section *Trichoderma*, clade *Viride, Koningii* aggregate, as demonstrated by Samuels et al. (2006). In this context, constructing a phylogenetic tree with reference sequences for the *CaM* region allowed the alignment of P05R2 to the clade comprising *T. koningiopsis* strains (Supplementary Figure S5).

For bacteria, sequence similarity searches in the NCBI GenBank databases revealed the presence of cultivable members from various genera under the phyla *Proteobacteria* and *Firmicutes* (Table 3). *Burkholderia* was the most abundant genus among the isolates (41.67%), followed by *Bacillus* (25%). Regarding the distribution of strains at the collecting sites, both areas (P05 and P10) exhibited bacteria from the genera *Burkholderia* and *Bacillus*, sharing some common species such as *Burkholderia cepacia* and *Bacillus cereus*.

Table 3. Bacterial species identified through sequencing of partial regions of the 16S rRNA gene.

Strain	**Closest match at NCBI (%)**	**Access number (GenBank)**
	16S rRNA	*16S rRNA*
P05R8	*Bacillus cereus* (97.19%)	PP952416
P05R9	*Burkholderia cepacia* (98.51%)	PP952605
P05R10	*Bacillus* sp. (99.33%)	PP952417
P05R11	*Stenotrophomonas maltophilia* (100%)	PP952606
P05R12	*Burkholderia* sp. (99.08%)	PP952607
P10R13	*Dyella japonica* (99.71%)	PP952608
P10R14	*Paraburkholderia* sp. (100%)	PP952609
P10R15	*Burkholderia cepacia* (100%)	PP952610
P10R16	*Burkholderia* sp. (100%)	PP952611
P10R17	*Burkholderia cepacia* (98.98%)	PP952612
P10R18	*Bacillus cereus* (99.22%)	PP952613
P10R19	*Serratia marcescens* (98.95%)	PP952418

In area P10, three distinct bacterial representatives were identified, including *Dyella japonica*, *Paraburkholderia* sp., and *Serratia marcescens*. Meanwhile, in area P05, unique representatives included *Bacillus* sp. and *Stenotrophomonas maltophilia*. The analysis of the

dendrogram generated by the BOX-PCR technique (Figure 1) confirmed the existence of different bacterial groups, forming five clusters at 58% similarity. These results, combined with data from the NJ phylogenetic tree (Supplementary Figure S6) provided conclusive characteristics for distinguishing some of the formed groups. Among these, particular distinctions were noted in strain P10R19, identified as *Serratia marcescens*. This strain not only clustered with its type species branch in the phylogenetic tree, but also exhibited unique characteristics in the BOX-PCR analysis, being allocated to a distinct taxon compared to the others. Another significant point was that strain P05R11, identified as *Stenotrophomonas maltophilia*, showed an exclusive profile in the BOX-PCR, being allocated to a distinct clade in the phylogenetic tree. Strains P05R8 and P10R18, identified both as *Bacillus cereus*, were grouped on the same branch in both the BOX dendrogram and the NJ tree, confirming the species correlation. While strains P05R9 (*Burkholderia cepacia*) and P05R10 (*Bacillus sp.*) presented similar profiles in the BOX-PCR, they were distinguishable via the NJ tree. Strains P05R12 and P10R15, identified as *Burkholderia* sp. and *Burkholderia cepacia*, shared the same branch in the BOX-PCR analysis, but were differentiated by their grouping into distinct taxa within the same clade in the phylogenetic tree. For strains P10R14 and P10R16, identified as *Paraburkholderia* sp. and *Burkholderia* sp., no significant differences were observed in the BOX-PCR profile. However, the phylogenetic interactions displayed by the NJ tree were conclusive in distinguishing the species.

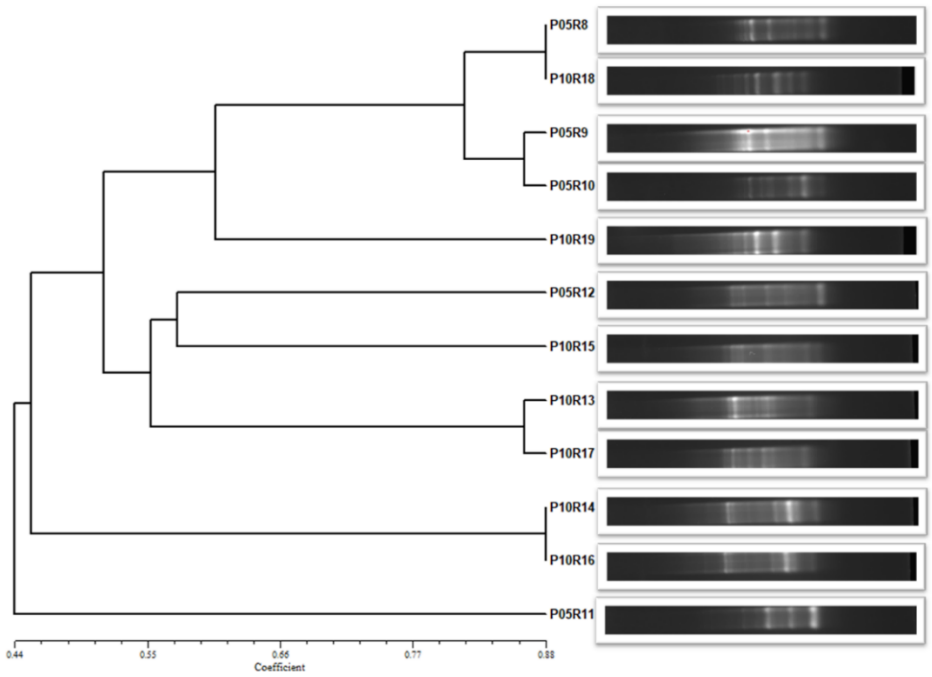

Figure 1. Dendrogram constructed from the BOX-PCR data profile of 12 bacterial isolates. The UPGMA method was used based on the similarity matrix. At 58% similarity the isolates were grouped into 5 clusters.

4.3.4. Screening for Biosurfactants

A total of 19 isolates, comprising 7 fungi and 12 bacteria, were screened for biosurfactant production in minimal salt medium (MSM) using soybean oil as the sole carbon source. Surface tension reduction (STR) was the parameter used to select strains for subsequent stages, as an increase in STR is considered indicative of surfactant activity (Da Silva et al., 2021). Based on our analysis, the average surface tension for the control medium was 54.57 $mN \cdot m^{-1}$. The supernatant of all fungal cultures demonstrated significant surfactant capabilities, evidenced by an STR > 20% (Figure 2).

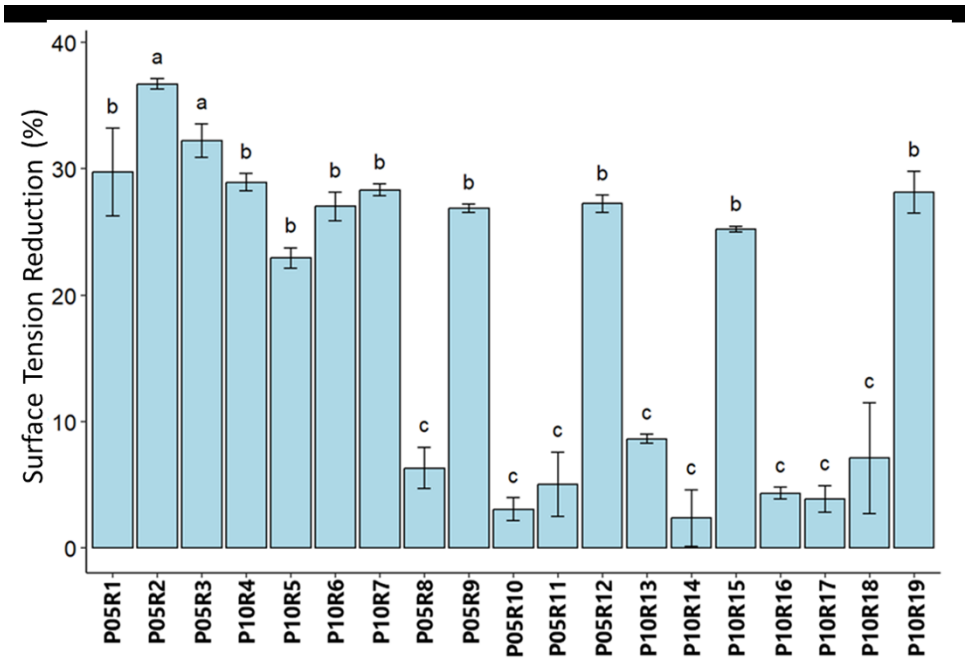

Figure 2. Results of screening for biosurfactant-producing isolates; *Classification into groups according to Scott-knott statistical analysis; *p-value* < 0.0001.

Among the bacterial strains, only four showed relevant surfactant activity. For fungi, the strain identified as *T. koningiopsis* P05R2 stood out by reducing the medium's surface tension by 36.6%. From the three best results, strains P05R1 and P05R3 identified as *P. janthinellum* stood out, with an STR of 29.7% and 32.2%, respectively. Among the four bacterial representatives that demonstrated significant surfactant activity, strain *S. marcescens* P10R19 had the highest STR (28.1%). This was followed by strains of the genus *Burkholderia*

(P05R9, P05R12, and P10R15), which reduced the medium's surface tension by 26.8%, 27.2%, and 25.1%, respectively.

4.3.5. Screening for Laccases

Preliminary screening of all isolated strains revealed a greenish-blue color shift, characteristic of laccase presence, in four fungal (P05R1, P05R2, P05R3, and P10R5) and five bacterial strains (P05R8, P05R9, P05R11, P10R16, and P10R19). Only those isolates exhibiting a positive color change in this initial assay were selected for subsequent quantitative analysis of extracellular laccase activity. Among the fungal isolates selected, the quantitative assay detected the first sign of enzymatic activity after three days of culture, exhibited by *P. janthinellum* P05R3 (0.167 U/mL) (Figure 3).

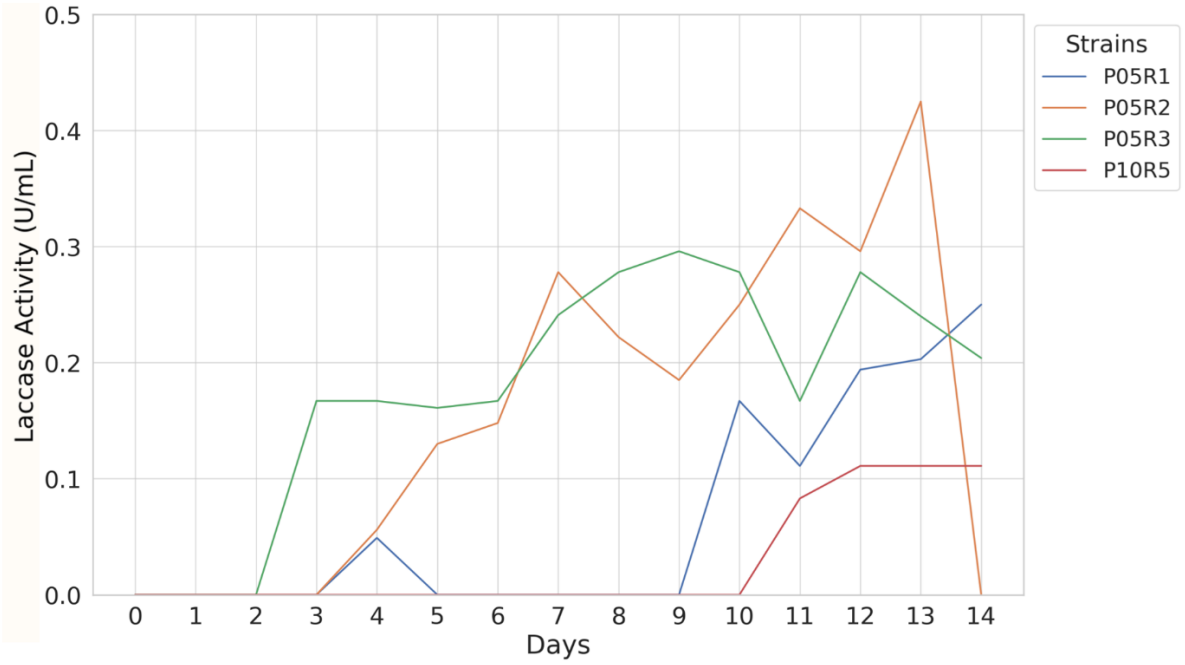

Figure 3. Monitoring extracellular laccase activity for fungal species.

On the fourth day of monitoring, other strains also showed evidence of enzymatic activity, including *P. janthinellum* P05R1 (0.049 U/mL) and *T. koningiopsis* P05R2 (0.056 U/mL). Overall, alternating peaks of activity were observed starting from the seventh day of cultivation. Among the fungi analyzed, *T. koningiopsis* P05R2 exhibited the highest enzymatic activity with a peak of 0.425 U/mL on the thirteenth day. During the monitoring period, two

other peaks of enzymatic activity were observed prior to the maximum value for this strain: on the seventh day (0.278 U/mL) and on the eleventh day (0.333 U/mL). Additionally, significant signals were detected for *P. janthinellum* P05R3 on the ninth day, reaching 0.296 U/mL. The strains *P. janthinellum* P05R1 and *P.* sp. P10R5 showed later signs of enzymatic production, reaching maximum peaks on the fourteenth day, with 0.250 U/mL and 0.111 U/mL, respectively.

For bacteria, the enzymatic peak for all strains was reached within the first 24 hours, as show in Figure 4. Among the strains evaluated, *S. maltophilia* P05R11 stood out exhibiting a maximum enzymatic activity of 0.917 U/mL, followed by *S. marcescens* P10R19 with an activity of 0.694 U/mL. Other strains, including *B. cepacia* P05R9 (0.639 U/mL), *B. cereus* P05R8 (0.583 U/mL), and *Bacillus* sp. P10R16 (0.556 U/mL), also showed significant enzymatic activity. The optical density analysis of the cultures revealed the extracellular production of laccase throughout the growth phase, peaking during the exponential growth phase (12–24 hours), followed by an abrupt decline in enzymatic activity as the cultures entered the stationary growth phase (48-72 hours). Additional information regarding these findings is provided in Supplementary Table S1 and S2.

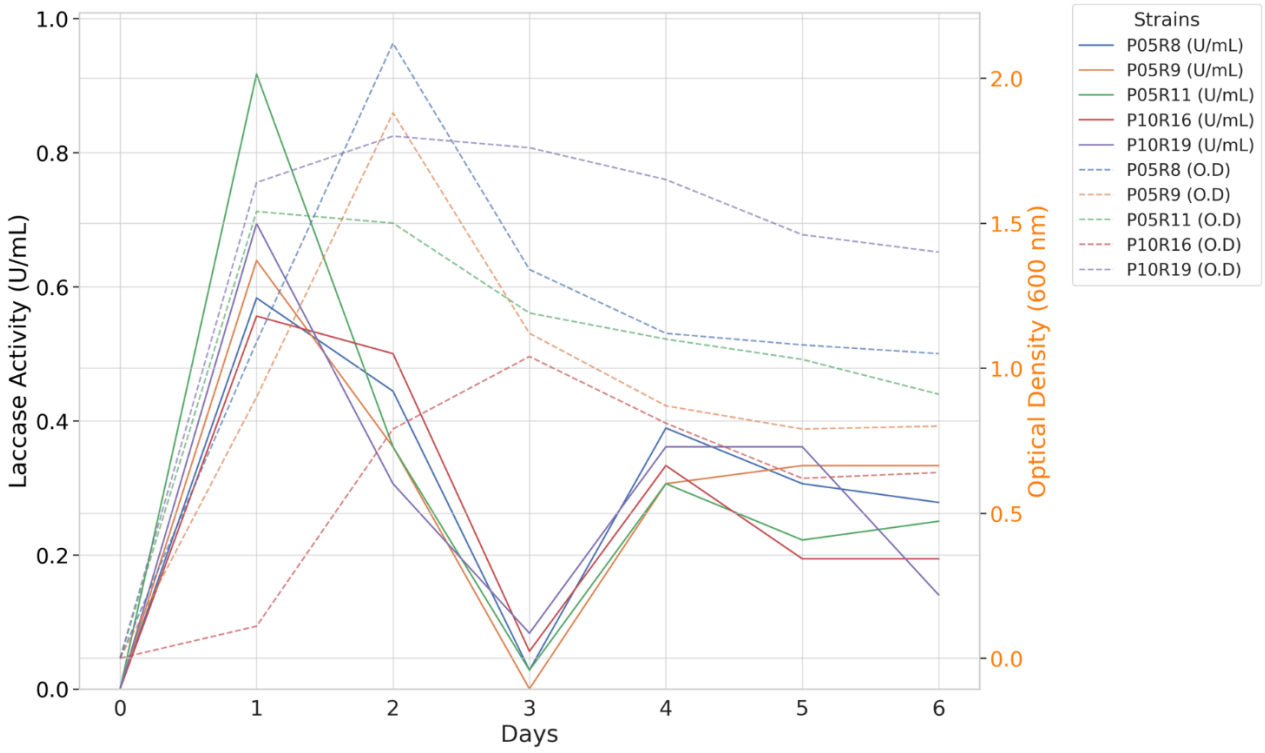

Figure 4. Monitoring laccase extracellular activity and microbial growth for bacterial species. Comparison of enzymatic activity relative to bacterial growth, measured by a spectrophotometer at 600 nm.

4.3.6. Selection and Assembly of Microbial Consortia

The analysis of the growth dynamics among the species selected for this screening phase revealed microbial interactions resulting in moderate antagonistic effects (+), partially inhibiting colony development, as well as more intense antagonistic activities (+ +), which led to complete inhibition of culture growth (Table 4). Based on this, different combinations were proposed to formulate compatible microbial consortia by selecting species that demonstrated the ability to grow together in minimal medium supplemented with 1% v/v diesel and did not exhibit antagonistic interactions (-). Combined with the previous data on biosurfactant and laccase production, the following consortia were formulated: mixed FFB1 (*Trichoderma koningiopsis* P05R2*, Penicillium janthinellum* P05R3, and *Serratia marcescens* P10R19), bacterial BB1 (*Stenotrophomonas maltophilia* P05R11 and *S. marcescens* P10R19), fungal FF1 (*T. koningiopsis* P05R2 and *P. janthinellum* P05R3), and mixed FBB1 (*T. koningiopsis* P05R2, *S. marcescens* P10R19, and *Burkholderia cepacia* P05R9).

Table 4. Assessment of antagonism activity between strains.

Bacteria vs. Fungus		**Bacteria vs. Bacteria**		**Fungus vs. Fungus**	
Strains	**Inhibition**	**Strains**	**Inhibition**	**Strains**	**Inhibition**
P05R1–P05R8	(−)	P05R9–P05R8	(−)	P05R1–P05R2	(−)
P05R1–P05R9	(+)	P05R9–P05R11	(+)	P05R1–P05R3	(+)
P05R1–P05R11	(+)	P05R9–P05R12	(+)	P05R1–P10R5	(+ +)
P05R1–P05R12	(−)	P05R9–P10R13	(−)	P05R1–P10R7	(+ +)
P05R1–P10R13	(−)	P05R8–P05R11	(+)	P05R2–P05R3	(−)
P05R1–P10R19	(−)	P05R8–P10R13	(−)	P05R2–P10R5	(+)
P05R2–P05R8	(−)	P05R11–P10R13	(−)	P05R2–P10R7	(+)
P05R2–P05R9	(−)	P05R12–P05R8	(+)	P05R3–P10R5	(−)
P05R2–P05R11	(+)	P05R12–P05R11	(−)	P05R3–P10R7	(−)
P05R2–P05R12	(+ +)	P05R12–P10R13	(−)	P10R5–P10R7	(−)
P05R2–P10R13	(−)	P10R19–P05R9	(−)		
P05R2–P10R19	(−)	P10R19–P05R12	(−)		

Table 4. Continuation

Bacteria vs. Fungus		Bacteria vs. Bacteria		Fungus vs. Fungus	
Strains	**Inhibition**	**Strains**	**Inhibition**	**Strains**	**Inhibition**
P05R3–P05R8	(+)	P10R19–P05R8	(−)		
P05R3–P05R9	(+)	P10R19–P05R11	(−)		
P05R3–P05R11	(+)	P10R19–P10R13	(−)		
P05R3–P05R12	(+)				
P05R3–P10R13	(−)				
P05R3–P10R19	(−)				
P10R5–P05R8	(+)				
P10R5–P05R9	(+ +)				
P10R5–P05R11	(−)				
P10R5–P05R12	(+ +)				
P10R5–P10R13	(−)				
P10R5–P10R19	(−)				
P10R7–P05R8	(−)				
P10R7–P05R9	(+ +)				
P10R7–P05R11	(−)				
P10R7–P05R12	(+)				
P10R7–P10R13	(+)				
P10R7–P05R16	(−)				
P10R7–P10R19	(−)				

The absence of inhibition zones or antagonistic effects between co-cultures is represented by (-); Moderate antagonistic effects are represented by (+); Strong antagonistic effects resulting in complete inhibition are represented by (+ +).

4.3.7. Biodegradation of Diesel Oil by Individual Strains and Microbial Consortia

After 15 days of incubation in MSM medium, the degradation rates of total hydrocarbons and PAHs present in the remaining diesel oil differed greatly between treatments (Figure 5). An exception was the 4-bromofluorobenzene and chrysene, which were 99.90% and

100% degraded by all strains and consortia combinations. Compared to the control samples, the individual bacterial strain *S. marcescens* P10R19 exhibited high removal rates for 2–4 ring PAHs, including pyrene (92.08%), anthracene (82.60%), 1-methylnaphthalene (93.80%), fluoranthene (83.14%), and acenaphthylene (84.65%), demonstrating superior capability compared to other strains, such as *S. maltophilia* P05R11, which removed pyrene (51.89%), anthracene (53.59%), 1-methylnaphthalene (49.83%), fluoranthene (43.80%), and acenaphthylene (60%). Additionally, the *S. marcescens* P10R19 strain was able to degrade 77.77% of the TPH (C5–C40) and 74.48% of total PAHs, whereas *S. maltophilia* P05R11 degraded 45.95% of TPH (C5–C40) and 36.60% of total PAHs. When these two bacterial strains were combined in the BB1 consortium, significantly higher degradation percentages were observed, achieving 87.05% for total PAHs and 93.97% for TPH (C5–C40). The consortium showed improved removal rates for hydrocarbons such as naphthalene (99.95%), fluorene (73.15%), and phenanthrene (66.67%), surpassing the degradation capabilities of the individual strains.

Among the individually evaluated fungi, *P. janthinellum* P05R3 exhibited high degradation rates for total PAHs (83.16%) and TPH (C5–C40) at 82.53%, effectively removing hydrocarbons such as naphthalene (97.25%), acenaphthene (86.65%), and 1-methylnaphthalene (93.71%). In contrast, *T. koningiopsis* P05R2 achieved degradation rates greater than 60% only for specific hydrocarbons like 1-methylnaphthalene (88.74%). The combination of these two fungal strains in the FF1 consortium resulted in enhanced degradation rates, achieving 88.77% removal of total PAHs and 92.08% of TPH (C5–C40).

The mixed fungal-bacterial consortium FFB1, composed of *T. koningiopsis* P05R2, *P. janthinellum* P05R3 and *S. marcescens* P10R19, removed 90.41% of total PAHs and 83.77% of TPH (C5–C40). It achieved higher degradation rates for fluoranthene (99.03%) and fluorene (94.89%) compared to the BB1 and FF1 consortia. The consortium that exhibited the best results was FBB1, which included *T. koningiopsis* P05R2, *S. marcescens* P10R19 and *B. cepacia* P05R9 (the latter not evaluated individually). According to the heatmap presented in Figure 5, this consortium achieved degradation rates of ≥91% for all analyzed PAHs, indicating a wide spectrum of degraded compounds with significantly higher rates.

Overall, Figure 5 shows that the different consortia tested achieved removal rates of ≥83% for total PAHs and TPH (C5–C40), indicating significant removal across a broad range of hydrocarbons. Furthermore, after the assay, we observed that aliquots of the cultures, serially

diluted and plated, not only exhibited viability, but also showed no signs of antagonistic interactions between the strains within each consortium.

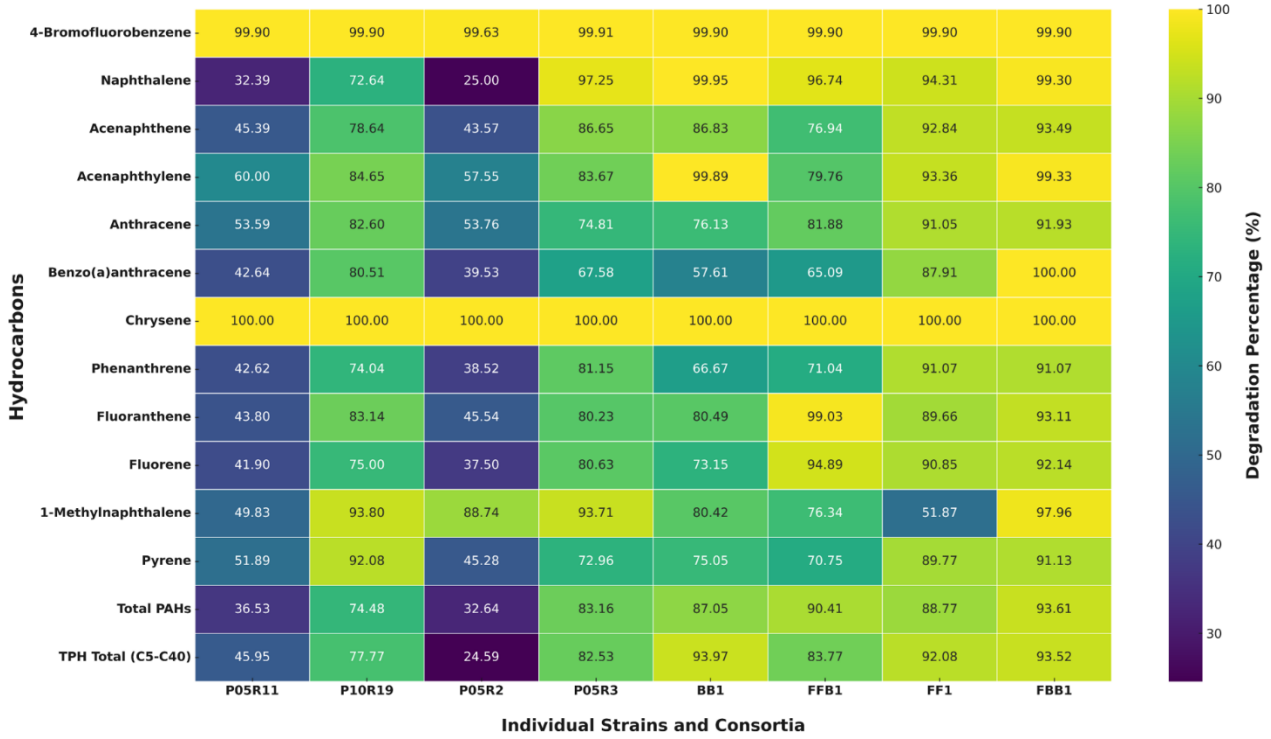

Figure 5. Efficiency of hydrocarbon degradation by individual strains and consortia. *P-value* < 0.05 according to ANOVA statistical analysis; Mixed fungi-bacteria consortia FFB1 (*Trichoderma koningiopsis* P05R2, *Penicillium janthinellum* P05R3, and *Serratia marcescens* P10R19), bacteria consortia BB1 (*Stenotrophomonas maltophilia* P05R11 and *S. marcescens* P10R19), fungi consortia FF1 (*T. koningiopsis* P05R2 and *P. janthinellum* P05R3), mixed bacteria-fungi consortia FBB1 (*T. koningiopsis* P05R2, *S. marcescens* P10R19 and *Burkholderia cepacia* P05R9), individual fungal strain *T. koningiopsis* P05R2, individual fungal strain *P. janthinellum* P05R3, individual bacterial strain *S. marcescens* P10R19, and individual bacterial strain *S. maltophilia* P05R11.

4.4. DISCUSSION

A remarkable persistence of hydrocarbons was detected in area P05 after more than a decade since the original release. Concentrations above the limits established by the regulatory governmental agency were detected. These analyses reinforce the perspective that, although biodiesel is readily biodegradable, mixed compositions of diesel/biodiesel pose an imminent risk to the environment. In a study in the same study area, Ramos et al. (2016) reported concentrations of benzene and benzo(a)pyrene above the regulatory values established after a period of 6.2 years since the experimental release of B20 diesel blends. According to the authors, these effects were attributed to the rapid consumption of electron acceptors, stimulated by the presence of biodiesel, forming an anaerobic degradation area, which delayed the removal

of remaining mass. As pointed out by the literature, the metabolic pathways used by microorganisms during the transformation and degradation of hydrocarbon require a substantial amount of energy and predominantly occur under aerobic conditions (Pandey; Pathak; Dave, 2016; Thacharodi et al., 2023). In area P10, for instance, the bio-stimulation of native bacteria, through supplementation with iron oxides and ammonium acetate, provided significantly lower levels of benzene compared to the monitored natural attenuation of area P05 (Müller et al., 2017). Indeed, our analysis found that benzene levels in area P10 were undetectable. However, the concentration of hydrocarbons such as xylenes, toluene, naphthalene, and anthracene remained above the standard levels.

Throughout the investigation, we found that among the identified fungal species, *Trichoderma koningiopsis* and *Penicillium pulvillorum* had not been previously described in areas impacted by petroleum hydrocarbons, making this the first report of these species in oil contaminated sites. The predominance of Ascomycetes in this initial analysis is consistent with findings from other studies that report the widespread distribution of genera like *Aspergillus*, *Penicillium*, and *Trichoderma* in hydrocarbon contaminated areas (Ferreira; Varjani; Taherzadeh, 2020; Horel; Schiewer, 2020; Medaura et al., 2021). Characteristics such as tolerance to adverse environmental conditions, production of multi-enzyme complexes and surfactant molecules, exhibited by some species within these genera, contribute to their establishment in different habitats, promoting the transformation and degradation of a wide range of hydrocarbons (Pandey; Pathak; Dave, 2016; Da Silva et al., 2023; Narayanan; Ali; El-Sheekh, 2023).

In agreement with the present study, Covino et al. (2015) reported that several Ascomycetes, including strains of *Fusarium*, *Penicillium,* and *Aspergillus*, were able to degrade approximately 79% of the aliphatic hydrocarbons present in contaminated soils. Another study of fungal communities in oil-contaminated sediments also demonstrated the role of species like *A. terreus*, *P. citreonigrum*, and *T. harzianum* in the degradation of aliphatic hydrocarbons (Bovio et al., 2017). Furthermore, an intervention in diesel-contaminated soil revealed that the high enzymatic activity, exhibited by fungal communities, was responsible for the degradation of 64% of four-ring PAHs, 28% of five-ring PAHs, 21% of two–three-ring PAHs and 16% of six-ring PAHs (Borowik; Wyszkowska; Oszust, 2017). Although not reported for bioremediation, species such as *T. koningiopsis* are well-known for their use in the biocontrol of phytopathogens, including *Fusarium*, and in promoting plant growth through phosphate

solubilization (Tandon et al., 2020; Yu; Luo, 2020; Ulrich et al., 2021). Therefore, the potential of the species in hydrocarbon degradation had not yet been explored.

With respect to bacteria, rapid development of strains was observed during the initial stages of in vitro sample enrichment, resulting in a greater number of isolates compared to fungi. In addition to the high concentration of hydrocarbon contaminants in the collecting areas, another factor that may have influenced the number of isolates includes the availability of oxygen and nutrients, which decreases with depth (Al-Turki, 2009; Aydin et al., 2017). In this study, we found a prevalence of species from the genus *Burkholderia*, which was similarly documented by Ramos et al. (2014) in experimental areas adjacent to P05 and P10. According to the findings of those authors, despite the predominance of *Burkholderia* in areas contaminated with B20 diesel, insignificant removal of BTEX and PAHs was detected during the period this bacterial species was dominant. It can be inferred that the proliferation of *Burkholderia* was associated with the consumption of acetate or methyl esters from the biodiesel. Somtrakoon et al. (2008), on the other hand, associated *Burkholderia* (strain VUN10013) isolated from petrochemical-contaminated areas, with the removal of PAHs (fluoranthene and pyrene). In addition to these, more than 79 bacterial genera have been associated with the degradation of hydrocarbons, including *Achromobacter*, *Acinetobacter*, *Alkanindiges*, *Alteromonas*, *Enterobacter*, *Arthrobacter*, *Bacillus*, *Dietzia*, *Kocuria*, *Marinobacter*, *Mycobacterium*, *Pandoraea*, *Pseudomonas*, *Staphylococcus*, *Rhodococcus*, *Stenotrophomonas*, and *Serratia*, among others (Sierra-Garcia et al., 2013; Imron et al., 2020; Patel et al., 2020).

Among the species screened for biosurfactant production, *T. koningiopsis* P05R2 stood out, reducing the surface tension of the medium by 36.6%. This value was 3.3% higher than the threshold proposed by Walter, Syldatk, and Hausmann (2010) for the selection of efficient biosurfactant-producing species, suggesting that *T. koningiopsis* P05R2 may be a promising species for this purpose. Although species from genera like *Trichoderma* and *Penicillium*, such as *T. citrinoviride*, *T. harzianum*, *T. atroviride*, *T. reesei*, *T. camerunense* (Martinho et al., 2019; Pitocchi et al., 2020; Piegza et al., 2021), *P. islandicum*, *P. commune,* and *P. citrinum* (Al-Hawash; Zhang; Ma, 2019; Kulkarni; Nene; Joshi, 2020; Kumar et al., 2023), have been documented for biosurfactant production, there is an evident gap in the literature concerning studies that investigate the relationship between the production of biosurfactants by these genera and the enhanced hydrocarbon biodegradation. In a recent review, Rani et al. (2024) also highlighted the scarcity of studies linking fungal biosurfactant production to hydrocarbon

degradation and emphasized the need for more research in the field. While our study focused only on a preliminary screening for biosurfactant production, the results obtained, combined with the analysis of hydrocarbon degradation, provide novel insights into this underexplored area, offering additional information about these interactions and their potential.

Contrary to the case with fungi, the connection between biosurfactant-producing bacteria and enhanced hydrocarbon degradation has been documented by numerous authors (Bezza; Chirwa, 2017; Gupta et al., 2020; Ray et al., 2021; Rehman et al., 2021). A good example is *S. marcescens* P10R19, which had an STR of 28.1%. Using a different strain of the same species, Zhang et al. (2021) demonstrated its capability of effectively degrading hydrocarbons while simultaneously producing biosurfactants. Other studies have also linked the emulsifying ability of *S. maltophilia* with enhanced degradation of PAHs (Tripathi et al., 2020). In this regard, we identified *S. maltophilia* P05R11, a similar species that, despite its low STR of 5%, could be used for this purpose. It is also important to consider that, as this screening aimed at selecting candidates for bioremediation, optimal parameters for the production of these molecules were not evaluated. Therefore, the STR percentages achieved by the strains can likely be improved through further optimizations.

The monitoring of laccase activity revealed that all five bacterial strains evaluated reached peak enzyme production within the first 24 h, with significantly higher values compared to the fungal strains. This rapid production and higher activity are essential for efficient bioremediation, as it is well known that these enzymes catalyze the simultaneous oxidation of a large number of aromatic compounds and are directly responsible for degrading various PAHs (Giardina et al., 2010; Thacharodi et al., 2023). However, most research for these applications focuses on laccases produced by fungi, citing the low expression and catalytic efficiency of bacterial laccases as justification (Bertrand; Martínez-Morales; Trejo-Hernández, 2017; Agarwal et al., 2022). Consequently, few studies cover the correlation between the production of these enzymes during bacterial growth and enhanced hydrocarbon degradation, including PAHs (Zerva et al., 2019; Agarwal et al., 2022).

Recent research has explored the engineering of bacterial laccases through methods such as directed evolution and heterologous functional expression (Wang et al., 2022; Narayanan; Ali; El-Sheekh, 2023). In this respect, genetically engineered strains of *E. coli*, such as BL21, have been used to overexpress laccase, achieving an enzyme activity of 9.5 U/mL. This laccase was utilized to enrich bacterial consortia composed of *Brevibacillus* sp. DL-1, *Bacillus* sp. DL-

13, and *Acinetobacter schindleri* DL-34. The laccase-enriched consortia showed improved crude oil degradation efficiency, achieving 68.2% compared to 60.6% without the added laccase (Dai et al., 2021). These results demonstrate superior enzymatic activity compared to *S. maltophilia* P05R11, the best-producing strain found in the present study, which achieved yields of 0.917 U/mL. Another approach more commonly found in the literature for the production of bacterial laccases is the optimization of cultivation parameters (Muthukumarasamy et al., 2015; Rajeswari M; Vennila K; Bhuvaneswari V, 2015; Neifar et al., 2016). Following this, Galai; Limam and Marzouki (2009) and Muthukumarasamy et al. (2015) reported a peak enzymatic activity of 16 U/mL for the strain *S. maltophilia* AAP56 achieved after 24 h of cultivation. Using a similar methodology, Ali et al. (2022) observed a maximum activity of 0.0733 U/mL for *Serratia proteamaculans* AORB19 after 48 h of cultivation. In the current investigation, the strain from the same genera, identified as *S. marcescens* P10R19, reached levels of 0.694 U/mL.

Among the analyzed fungi, we observed alternating peaks of enzymatic activity over a monitoring period of 14 days. *Trichoderma koningiopsis* P05R2 exhibited the highest peak (0.425 U/mL) on the thirteenth day of trial. The observations recorded were similar to those of Hölker, Dohse, and Höfer (2002), who identified the first sign of enzymatic activity for a strain of *T. harzianum* on the fourth day of the culture, reaching maximum activity on the ninth day of cultivation (0.0138 U/mL). Similarly to the observations made for *T. koningiopsis* P05R2, a sharp decline in enzymatic activity started on the fourteenth day, rendering it undetectable after that.

In a similar study, a strain identified as *T. viride* isolated from soil samples contaminated with petroleum hydrocarbons, demonstrated the ability to produce extracellular laccases, reaching 23 U/mL. On the other hand, *Penicillium chrysogenum* showed an activity of 79 U/mL (Balaji; Arulazhagan; Ebenezer, 2014). The production of these enzymes by fungi from the genera *Trichoderma* and *Penicillium*, along with their associated hydrocarbon degradation, can be found in various reports in the literature, demonstrating the potential of these genera for bioremediation (Zafra; Cortés-Espinosa, 2015; Kadri et al., 2017; Zehra et al., 2018).

Finally, the adoption of these screening methodologies enabled the formulation of distinct microbial consortia, which demonstrated the ability to efficiently remove a broad spectrum of PAHs and the TPH (C5–C40) present in diesel oil. The results of our analyses revealed a significant contrast between the capacity of the strains evaluated separately and their

use in consortia, suggesting that the synergy between the strains was essential for the degradation of many hydrocarbon constituents. In agreement, numerous researchers highlight that the combined action of different microbial species, equipped with suitable metabolic mechanisms, can enhance the degradation efficiency and expand the range of compounds susceptible to microbial action (Ławniczak et al., 2020; Qian et al., 2020; Kapoore et al., 2022).

In the present study, the use of microbial consortia demonstrated enhanced hydrocarbon degradation capabilities compared to individual strains. The combination of *S. maltophilia* P05R11 and *S. marcescens* P10R19 in the BB1 consortium resulted in significantly higher degradation percentages for both total PAHs and TPH (C5–C40). The benefit of using a bacterial consortium composed of the biosurfactant-producing strains *B. cereus* and *B. pumilus* over individual strains for the degradation of TPH, with special emphasis on PAHs, was also reported by Patowary et al. (2016). After 5 weeks of incubation, the authors observed the following degradation rates for listed hydrocarbons: naphthalene (70.87%), fluorene (61.79%), phenanthrene (58.98%), anthracene (100%), and TPH C5–C40 (84.15%). In this regard, the capacity of the BB1 consortium for hydrocarbon removal was significantly superior when comparing the degradation results obtained for hydrocarbons such as naphthalene (99.95%), fluorene (73.15%), phenanthrene (66.67%), and TPH C5–C40 (87.05%).

In a more recent study, Tripathi et al. (2023) evaluated the degradation capabilities of individual bacterial strains of *Pseudomonas boreopolis* IITR108, *Microbacterium schleiferi* IITR109, *P. aeruginosa* IITR110 and *Bacillus velezensis* IITR111, which were isolated from crude oil. These strains showed 80–89% degradation of TPH (C8–C40) and 71–78% degradation of total PAHs. When these bacteria were combined into a consortium, they demonstrated degradation capabilities similar to those presented by the BB1 consortium, achieving 93.2% degradation of TPH (C8–C40) and 85.5% of total PAHs. Nnabuife et al. (2022), on the other hand, reported that the degradation of TPH by the bacterial strains *P. aeruginosa* W15, *Providencia vermicola* W8, and *Serratia marcescens* W13 was higher when the strains were evaluated individually. These findings demonstrate that potential antagonistic effects may occur depending on the composition of the consortia, impacting the ability to remove particular compounds. In the present study, in addition to the results being improved by using the strains in consortia, it was also observed that the consortia remained viable and did not display any halos indicative of antagonistic interactions after the degradation assays.

In comparison to the BB1 bacterial consortium, the combination of *T. koningiopsis* P05R2 and *P. janthinellum* P05R3 in the FF1 consortium demonstrated enhanced degradation of total PAHs. Previous studies have highlighted the efficacy of fungal consortia in hydrocarbon degradation, emphasizing the role of ligninolytic enzymes. For example, Khan (2015) reported that *P. janthinellum* was capable of degrading 96% of kerosene and 75% of diesel, with concomitant production of enzymes such as MnP and Lac, indicating that this species has a high potential for the application for bioremediation. Similarly, Vipotnik, Michelin, and Tavares (2021) observed that a fungal consortium composed of the laccase-producing species *Aspergillus aegerita* and *P. chrysogenum* was capable of degrading 86% of fluorene when used as the sole carbon source. In comparison, the fungal consortium FF1 achieved a higher degradation rate for the same hydrocarbon, reaching removal rates of 90.85%.

Our findings further demonstrate that the integration of fungi and bacteria in mixed consortia can substantially enhance hydrocarbon degradation. These results are consistent with studies that have highlighted the cooperative roles of fungi and bacteria in mutualistic relationships, which facilitate hydrocarbon degradation. Ghorbannezhad, Moghimi, and Dastgheib (2021) suggest that fungi play a primary role in the early stages of high-molecular-weight (HMW) PAHs and long-chain aliphatic degradation, while bacteria focus on removing the produced metabolites, completing the degradation process and thereby assisting fungi by reducing catabolic repression. The same authors also found that the addition of biosurfactants increased the degradation of pyrene and tetracosane by approximately 60.76% and 73.48%, respectively. Supporting the previous statement, Boochan et al. (2000) investigated the biodegradation of HMW PAHs in liquid media using the bacteria *S. maltophilia* and the fungus *P. janthinellum*. They found that when evaluated individually, the bacteria utilized pyrene as their sole source of carbon and cometabolically mineralized benzo[a]pyrene when pyrene was present. Significant degradation and microbial growth on pyrene, chrysene, benz[a]anthracene, benzo[a]pyrene, and dibenz[a,h]anthracene occurred when the strains were co-cultivated, indicating that the mutual interaction between the strains was responsible for the enhanced degradation.

Similarly, *T. koningiopsis* P05R2, evaluated for the first time in this context, was not able to effectively degrade hydrocarbons when tested individually. However, when combined with different bacterial strains, the degradation of a broad spectrum of PAHs and TPH (C5–C40) was achieved. In this way, the cooperative role of *T. koningiopsis* in the degradation process highlights and reinforces the strain's potential for future applications in microbial

consortia designed for bioremediation. In another study, a consortium composed of five fungal isolates (*Aspergillus flavus* H6, *A. nomius* H7, *Rhizomucor variabilis* H9, *Trichoderma asperellum* H15, and *A. fumigatus* H19) and seven bacterial isolates (*Klebsiella pneumoniae* B1, *Enterobacter* sp. B3, *B. cereus* B4, *P. aeruginosa* B6, *Streptomyces* sp. B8, *Klebsiella* sp. B10, and *S. maltophilia* B14) was able to remove 87.76% of phenanthrene, 48.18% of pyrene, and 56.55% of benzo[a]pyrene after 14 days (Zafra et al., 2014). Thus, we can emphasize that the consortia developed in the present study covered a broader spectrum of degraded hydrocarbons compared to many other reports that deal with the application of mixed consortia for hydrocarbon remediation.

4.5. CONCLUSIONS

This study explored the potential of individual strains and novel mixed microbial consortia, composed of fungal and bacterial species isolated from areas contaminated with blends of diesel B20 (20% biodiesel and 80% diesel), in the degradation of PAHs and TPH (C5–C40) present in diesel oil. Among the biosurfactant and laccase-producing species selected for analysis, *Trichoderma koningiopsis* was identified for the first time as a candidate for bioremediation of petroleum hydrocarbons. The assays demonstrated that while some species showed promising individual performance, the combination of some strains in mixed consortia resulted in significantly greater degradation. A good example is the combination of *S. maltophilia* P05R11 and *S. marcescens* P10R19, reaching degradation rates of 87.05% for total PAHs and 93.97% for TPH (C5–C40), far superior when compared to the individual capacities of each strain alone. Similarly, the combination of the fungal strains *P. janthinellum* P05R3 and *T. koningiopsis* P05R2 resulted in 88.77% degradation of total PAHs and 92.08% of TPH (C5–C40). Nevertheless, the consortium that exhibited the best results was a mixed bacterial–fungal consortium, composed of *T. koningiopsis* P05R2, *S. marcescens* P10R19, and *B. cepacia* P05R9. This consortium was the only one to cover a degradation spectrum ≥91% for all PAHs analyzed, removing 93.61% of total PAHs and 93.52% of TPH (C5–C40). These results indicate that the combined action of different microbial species, equipped with different metabolic mechanisms, can significantly enhance the efficiency of hydrocarbon degradation. Therefore, our findings corroborate the premise that mixed microbial consortia are more effective in hydrocarbon remediation than isolated strains, covering a broader spectrum of degraded compounds. Future research should focus on optimizing these consortia and their

application in contaminated soil microcosms to understand the extent of their degradation potential and behavior across different matrices.

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5. CHAPTER III

MICROBIAL BIOREMEDIATION: REFLECTIONS, CONTRIBUTIONS, AND FUTURE HORIZONS

5.1. Final Considerations

The journey from microbial screening to the optimization of mixed microbial consortia for the degradation of petroleum hydrocarbons represents a significant step forward in the quest for sustainable and efficient bioremediation strategies. This study has illuminated the intricate interplay between bacterial and fungal species, revealing their synergistic metabolic potential in breaking down persistent pollutants such as polycyclic aromatic hydrocarbons (PAHs) and total petroleum hydrocarbons (TPH). Beyond the empirical data and quantitative degradation efficiencies, the broader implications of this research extend to scientific innovation, environmental restoration, and the evolving paradigm of energy sustainability.

5.2. A Holistic Overview

The convergence of microbial biotechnology and environmental science within this research underscores the intricate balance between nature's resilience and human intervention. The findings not only validate the efficacy of microbial consortia in degrading complex hydrocarbon compounds but also reinforce the notion that nature itself holds solutions to the very problems induced by industrial activities. The ability of these microorganisms to adapt, evolve, and cooperate presents an invaluable blueprint for developing bio-based technologies that align with ecological equilibrium.

Furthermore, the identification of *Trichoderma koningiopsis* as a candidate for hydrocarbon bioremediation exemplifies the untapped potential within microbial diversity. It reinforces the imperative of continuous bioprospecting, where new species and metabolic pathways can be harnessed for environmental applications. The broader scientific contributions of this study lie not only in its empirical findings but also in its methodological framework, an approach that integrates strain selection, compatibility testing, and degradation assays to optimize microbial consortia for targeted bioremediation applications.

5.3. Contributions and Future Perspectives

From an applied standpoint, this research contributes directly to the petroleum and gas industries by offering a viable alternative for mitigating hydrocarbon contamination. The development of efficient microbial consortia represents a paradigm shift from traditional physicochemical remediation methods, which often prove costly, inefficient, or

environmentally invasive. By harnessing the metabolic versatility of fungi and bacteria, this work provides a foundation for the formulation of next-generation bioaugmentation strategies tailored to contaminated environments.

Moreover, these findings prompt crucial discussions regarding the future of microbial-assisted remediation. What other microbial interactions remain unexplored? How can these findings be scaled to field applications with real-world contamination challenges? These questions serve as catalysts for continued research and innovation, urging the scientific community to refine and optimize the application of microbial consortia across diverse environmental matrices.

5.4. Innovation and Opportunities

The innovation embedded within this study is twofold: it lies both in the scientific methodology employed and in the broader environmental and industrial applications derived from these findings. By demonstrating that mixed microbial consortia significantly outperform individual strains in hydrocarbon degradation, this research reinforces the necessity of strategic microbial selection and consortium formulation. The potential applications extend beyond bioremediation to fields such as industrial biotechnology, waste management, and bioenergy, where microbial metabolism can be further leveraged for sustainable solutions.

Additionally, this research opens doors for interdisciplinary collaboration. The intersection of microbiology, environmental engineering, and petroleum chemistry presents fertile ground for new technological advancements. The prospect of bioengineered consortia, synthetic microbial communities, and real-time monitoring of biodegradation efficiency using omics-based approaches could further refine and elevate the impact of this work. The incorporation of computational models to predict microbial interactions and degradation pathways also represents an avenue for future exploration.

5.4. Final Reflections

At its core, this research embodies the inherent potential of nature’s microscopic architects, microorganisms, as pivotal agents of change. The resilience, adaptability, and cooperative nature of microbial consortia serve as a metaphor for scientific progress itself,

where interdisciplinary collaboration and methodological refinement drive innovation. In bridging fundamental microbiology with practical environmental applications, this study reaffirms that solutions to contemporary industrial challenges often reside in the biological mechanisms that have evolved over millennia.

As we look ahead, the responsibility to harness these microbial capabilities in an ethically and ecologically sound manner becomes paramount. The intersection of scientific discovery and environmental stewardship compels us to continuously refine our approaches, ensuring that technological advancements align with sustainability and long-term ecological balance.

In the broader landscape of petroleum, gas, and bioenergy research, this work represents both a culmination of efforts and a launching point for new inquiries. The knowledge generated herein serves not as a final destination, but as a stepping stone towards more refined, efficient, and ecologically sound biotechnological interventions. With each discovery, we inch closer to a future where microbial bioremediation is not merely an alternative but a standard practice in environmental restoration, proving that the smallest organisms can yield the most profound impacts on our planet's health and sustainability.