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CAMILA PAIM DANTAS

PROSPECÇÃO DE ENZIMAS A PARTIR DE CONSÓRCIO MICROBIANO DEGRADADOR DE PETRÓLEO

Salvador
2022

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Tese apresentada ao Programa de Pós-Graduação em Geoquímica: Petróleo e Meio Ambiente – Pospetro, Instituto de Geociências, Universidade Federal da Bahia, como requisito para a obtenção do título de Doutor em Geoquímica do Petróleo e Ambiental.

Orientadora: Profa. Dra. Olívia Maria Cordeiro de Oliveira
Orientadora: Profa. Dra. Danusia Ferreira Lima

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CAMILA PAIM DANTAS

PROSPECÇÃO DE ENZIMAS A PARTIR DE CONSÓRCIO MICROBIANO DEGRADADOR DE PETRÓLEO

Tese apresentada como requisito parcial para obtenção do grau de Doutora em Geoquímica do Petróleo e Ambiental, Instituto de Geociências da Universidade Federal da Bahia.

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“Não é sobre chegar no topo do mundo e saber que venceu.

É sobre escalar e sentir que o caminho te fortaleceu [...]”

Ana Vilela

DANTAS, Camila. Paim. **Prospecção de enzimas a partir de consórcio microbiano degradador de petróleo.** 2022. 265 f. Tese (Doutorado em geoquímica: petróleo e meio ambiente) – Instituto de Geociências, Universidade Federal da Bahia, Salvador, 2022.

RESUMO

A proteômica é considerada uma ferramenta biotecnológica em ascensão que auxilia na compreensão dos mecanismos envolvidos na biotransformação/biodegradação de hidrocarbonetos. Assim sendo, este trabalho teve o intuito de prospectar os padrões enzimáticos diferenciais do consórcio microbiano degradador do petróleo da bacia do Recôncavo Baiano na presença do contaminante, para futuras aplicações em sistemas de recuperação de ambientes impactados por petróleo. O consórcio microbiano misto é composto por 10 bactérias e 23 fungos de origem marinha, os quais foram identificados pelo método Sanger utilizando os genes 16S e ITS. O consórcio foi submetido a agitação constante de 180 rpm a 35 °C durante 7 dias para avaliação da deplecção de óleo cru e a identificação dos padrões enzimáticos na presença do petróleo. A concentração dos hidrocarbonetos foi detectada por cromatografia gasosa acoplada a detector de ionização em chama conforme protocolo adaptado da USEPA 3510 e a degradação avaliada pelas razões Pr/Ph, Pr/C_{17}, Ph/C_{18}, HTP/UCM e outras. O sequenciamento dos peptídeos ocorreu em cromatografia líquida MS-MS e as enzimas identificadas através do *software* PatternLab for Proteomics. A partir dos resultados preliminares foi possível verificar que houve redução dos hidrocarbonetos resolvidos e hidrocarbonetos de mistura complexa não resolvida, bem como os isoprenóides, pristano e fitano. A análise proteômica revelou a predominância de enzimas relacionadas à resposta ao estresse, transporte e tradução/transcrição quando em presença de petróleo e também indicou a de uma biblioteca de proteínas relacionadas ao óleo bruto permite a compreensão do potencial de degradação do consórcio microbiano, bem como estabelece as bases para a otimização de um sistema mais robusto de recuperação ambiental.

Palavras-chave: Biocatálise, biorremediação, biodegradação, proteômica, HTP, HPA

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ABSTRACT

Proteomics is considered an emerging biotechnological tool that helps to clarify the mechanisms involved in the biotransformation/biodegradation of hydrocarbons. Therefore, this work aimed to determine the differential enzymatic patterns of the microbial consortium degrading oil from the Recôncavo Baiano basin, in addition, this work is relevant for future systems in recovery of oil-impacted environments. The mixed microbial consortium was composed of 10 bacteria and 23 fungi of marine origin, which were identified by the Sanger method using the 16S and ITS genes. The consortium was subjected to constant agitation at 180 rpm at 35 °C for 7 days to assess crude oil depletion and identify enzymatic patterns in the presence of oil. The concentration of hydrocarbons was detected by gas chromatography coupled to a flame ionization detector according to the protocol adapted from USEPA 3510 and the degradation was evaluated by the ratios Pr/Ph, Pr/C_{17}, Ph/C_{18}, HTP/UCM and others. The peptides were sequenced using MS-MS liquid chromatography and the enzymes were identified using the PatternLab for Proteomics software. From the preliminary results, a reduction in resolved hydrocarbons and unresolved complex mixture hydrocarbons, as well as isoprenoids, pristane and phytane, was observed and could be verified. The proteomic analysis revealed the predominance of enzymes related to the stress response, transport and translation/transcription when in the presence of oil and indicated the presence of enzymes associated with the degradation of xenobiotics, indicating their possible use in biotechnological applications. Thus, constructing a library of proteins related to crude oil clarifies the degradation potential of the microbial consortium and lays the foundations for the optimizing of a more robust system of environmental recovery.

Keywords: Biocatalysis, bioremediation, biodegradation, proteomics, HTP, HPA

SUMÁRIO

1 INTRODUÇÃO

Diante das questões ambientais no que diz respeito à gestão de áreas contaminadas por petróleo, existem vários métodos de limpeza. Entretanto, busca-se novos produtos ambientalmente viáveis, que não afetem o meio ambiente, que sejam de alta eficiência e apresentem mecanismos mais econômicos do que os convencionais. Nesse contexto, a biocatálise e biotransformação são considerados processos biotecnológicos em ascensão nos setores acadêmicos e industrial (LUCCHESE et al., 2012; RODRIGUES et al., 2014).

Vários países vêm sofrendo tragédias ecológicas com o vazamento de petróleo e derivados. Estima-se que nos últimos anos foram derramadas aproximadamente 165.000 toneladas de óleo no mundo, sendo que: 61 % desse montante são causados por alisões ou colisões, aterramentos, falhas no casco de navios, falhas nos equipamentos e incêndios e explosões; 33 % são causados por eventos climáticos e erro humano e; 6 % por eventos desconhecidos (ITOPF, 2020). Apesar dos grandes derramamentos (>700 mil t) terem reduzido globalmente nas últimas décadas, a ocorrência de vazamentos < 700 mil toneladas ainda é vivenciada em diversos locais do mundo (ITOPF, 2020). Recentemente no segundo semestre de 2019 foi registrado o mais extenso desastre ambiental na história do Brasil, onde aproximadamente 3000 km da costa foi afetada pelo óleo (OLIVEIRA et al., 2020). Segundo o IBAMA (2020) > 200 toneladas de material oleoso pesado e altamente intemperizado foram retirados do litoral brasileiro desde o estado do Maranhão ao Rio de Janeiro, em cinco meses de monitoramento. Impactos com essa magnitude geram danos incalculáveis a fauna e flora local, bem como a várias atividades econômicas como turismo, pesca artesanal, esportes náuticos e aquicultura, sem falar nos efeitos de longo prazo à saúde da população (SOARES et al., 2020). Vários meses após o desastre na costa brasileira ainda é possível encontrar altas taxas de naftaleno, fenantreno, fluoranteno, fluoreno e acenaftaleno nos corpos d'agua, em peixes e em bivalves (SOARES et al., 2021). Devido a esse acontecimento a segurança alimentar foi comprometida e em resposta as vendas de pescados na região caíram mais da metade, impactando fortemente a geração de renda local (ESTEVO et al., 2021). Em ocasiões dessa magnitude, as autoridades locais devem estar preparadas para fornecer não apenas respostas rápidas baseadas na ciência, mas também uma comunicação científica eficaz para a sociedade a fim de reduzir os impactos causados (MAGALHÃES et al., 2022).

Considerando que os hidrocarbonetos do petróleo são poluentes orgânicos persistentes (POPs) e podem provocar alterações nocivas ao meio ambiente e ao homem (ABDEL-SHAFY; MANSOUR, 2016), diversos métodos podem ser utilizados, tais como: oxidação química, nanoremediação, dessorção térmica, fitorremediação e biorremediação, a fim de remediar áreas contaminadas (MORAES; TEIXEIRA; MAXMINIANO, 2014). O uso de microrganismos na remoção de poluentes agrega viabilidade para biorremediação, uma vez que proporciona bons resultados, é de fácil aplicabilidade, tem menor custo e menor geração de poluentes secundários (ZHANG; WU; RE, 2020). Por outro lado, os métodos físico-químicos convencionais parecem ser tecnicamente e economicamente desafiadores (VARJANI; UPASANI, 2017).

Muitos microrganismos, tais como *Penicillium chrysogenum, Pseudomonas putida, Pseudomonas aeruginosa, Bacillus subtilis, Bacillus cereus, Aspergillus niger* e *Candida glabrata* possuem mecanismo para transformar diferentes componentes do petróleo (HUNDIWALE et al., 2021). Essa aptidão é vista tanto para degradação de hidrocarbonetos aromáticos quanto alcanos, alcanos lineares, isoalcanos, ou ainda com capacidade de degradar mais de um composto orgânico, tornando possível sua utilização na recuperação de ambientes contaminados por petróleo (JESUS et al., 2015; LIMA, 2016). Ainda que muitos microrganismos tenham essa habilidade comprovada raramente uma única espécie terá a capacidade de transformar ou reduzir um poluente a concentrações aceitáveis em um período curto de tempo. Partindo-se dessa premissa, a construção heterogênea de um consórcio pode apresentar vantagens na degradação. Consórcio microbiano é uma mistura de várias espécies microbianas que juntas possuem interações sinérgicas entre si, ou seja, são microrganismos que agem em cooperação realizando a máxima degradação possível de compostos indesejáveis (GUPTA; KUMAR; PAL, 2019). Os atributos de resiliência, robustez, resistência ao estresse e multifunção tornam os consórcios microbianos mais resistentes às perturbações ambientais do que as cepas isoladas (MACCHI et al., 2021), principalmente se forem associados a técnica de encapsulamento e adição de nutrientes. Dessa forma, consórcios microbianos são considerados mais eficientes em relação a culturas puras e, portanto, tornou-se um atrativo para processos de degradação de compostos complexos como os que compõem o petróleo.

As transformações de poluentes orgânicos são eficazes e ocorrerem devido os componentes enzimáticos presentes em microrganismos que são poderosos catalisadores, capazes de modificar extensivamente a estrutura e as propriedades toxicológicas dos contaminantes ou

transformar completamente moléculas orgânicas em produtos finais inorgânicos inócuos (MARCHUT-MIKOLAJCZYK et al., 2015). A utilização de enzimas tem se tornado um atrativo no desenvolvimento de processos ou metodologias de síntese orgânica, pois além de ser rápida e seletiva também obedece os princípios da química verde ambiental (CAPELETTO; OMORI, 2017). No que tange a utilização de enzimas microbianas para promover a degradabilidade do petróleo e seus derivados, as peroxidases e oxigenases têm sido estratégias eficazes. Lignina peroxidase, manganês peroxidase, epóxido hidrolases, citocromo P450 monooxigenase, dioxigenases, foram amplamente investigadas devido à sua capacidade de degradar hidrocarbonetos policíclicos aromáticos (AL-HAWASH et al., 2018). Abdelhaleem et al. (2019) constataram que protocatecol 3,4-dioxigenase e catecol 1,2-dioxigenase assumem um papel central na degradação de hidrocarbonetos. A deplecção do antraceno requer um complexo multienzimático formado por NADH oxidoreductase, enzimas dioxigenase e moléculas de ferredoxina. Por outro lado, as enzimas lacase secretadas por fungos auxiliam na oxidação de pireno e a manganês peroxidase atua na desmineralização de antraceno, fenantreno, pireno e fluoranteno (SARAVANAN et al., 2021). Em vista disso, a combinação de múltiplas enzimas específicas para cada composto do petróleo podem consumir diferentes intervalos de substrato e transfigura-se em uma ferramenta adequada para remediação (PEIXOTO; VERMELHO; ROSADO, 2011).

A tecnologia enzimática é um fato inovador empregado em diversos setores industriais, tais como na produção de detergentes, alimentos, têxteis, farmacêuticas, cosméticos, diagnósticos e química fina, contudo o cenário atual carece de estudos aprofundados (SANTOS, et al., 2016). Atualmente, acredita-se que existem sistemas enzimáticos para degradação de alcanos que ainda não foram caracterizados e que poderiam incluir novas proteínas não relacionadas com as conhecidas atualmente (ROJO, 2009). Além disso, para explorar os múltiplos sistemas de oxidação, ou sobreposição de substrato envolvidos na degradação de alcanos deve ser levada em consideração a alta diversidade enzimática, assim como investigar ecossistemas pouco estudados (VAN BEILEN; JAN; FUNHOFF, 2007; WENTZEL et al., 2007). Existe uma concentração de pesquisas na literatura voltada à investigação de condições ideais de funcionamento das enzimas, ao invés da aplicação de produtos biotecnológicos, demonstrando ter um aspecto incipiente no desenvolvimento de bioprodutos (BREDA et al., 2017). São necessários projetos que envolvam prospecção e avaliação do potencial destas enzimas, a durabilidade e efetividade em relação ao

tempo de produção, vias de degradação, sobre a genética molecular e a bioquímica das vias catabólicas, para obter misturas de enzimas com características desejáveis e por fim produzir biocatalizadores de interesse tecnológico (KADRI et al., 2017).

O aspecto conjunto de investigar a aplicação de consórcio microbiano em sistema de biotransformação de petróleo que possam gerar produtos de alto valor agregado, juntamente com a possibilidade de redução de resíduos secundários no processo de biorremediação, justificam plenamente este trabalho. E ainda considerando a toxicidade dos petroderivados provenientes dos derrames acidentais da indústria petrolífera, associado à crescente preocupação com os impactos ambientais desses e uma legislação ambiental cada vez mais rígida, elevam a relevância desse estudo.

2 OBJETIVOS

Com o propósito de alcançar o alvo do projeto, têm-se os objetivos abaixo descritos.

2.1 OBJETIVO GERAL

Prospectar os padrões enzimáticos diferenciais do consórcio microbiano degradador do petróleo da bacia do Recôncavo Baiano na presença do contaminante, a fim de compreender quais as principais enzimas e suas potencialidades para futuras aplicações em sistemas de recuperação de sítios impactados por óleo.

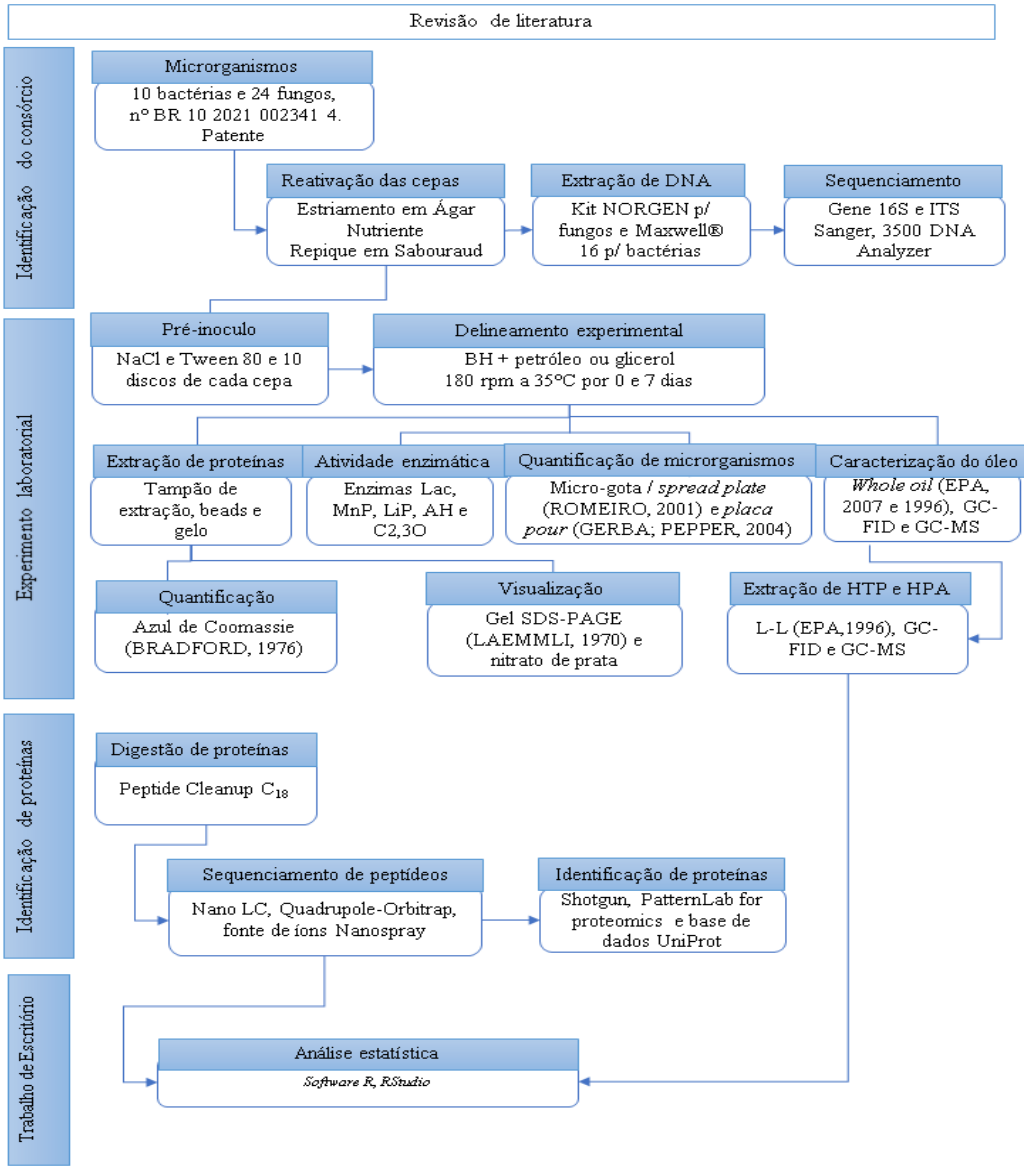
2.2 OBJETIVOS ESPECÍFICOS

- Identificar microrganismos provenientes de consórcio hidrocarbonoclástico por meio de ferramentas filogenéticas moleculares;
- Investigar a presença de microrganismos produtores de enzimas alcano hidroxilase, catecol 2,3 dioxigenases, lignina peroxidase, manganês peroxidase e lacase;
- Comparar a atividade enzimática das proteínas de interesse para degradação do petróleo em processo intempérico biótico e abiótico;
- Identificar as enzimas diferencialmente expressas na presença do petróleo da bacia do Recôncavo Baiano.

3 MATERIAL E MÉTODO

Para alcançar a proposta deste projeto foi utilizada a estrutura do Centro de Excelência em Geoquímica Petróleo, Energia e Meio Ambiente (LEPETRO) do Instituto de Geociências na Universidade Federal da Bahia (UFBA), do Laboratório de Entomologia Molecular (LENT-MOL) do Instituto de Ciências Biológicas na Universidade Estadual de Feira de Santana (UEFS) e do Instituto de Biotecnologia da Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP). O experimento foi conduzido através das etapas metodológicas da Figura 1.

Figura 1 – Fluxograma metodológico da pesquisa e suas etapas subsequentes



Fonte: Autora, 2022.

3.1 REVISÃO DE LITERATURA

O levantamento bibliográfico deste projeto foi realizado por meio de buscas de artigos científicos e livros (em português, espanhol e inglês) no portal de periódicos da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), assim como através do acervo da Biblioteca da Universidade Federal da Bahia, Google livros e Google Acadêmico para busca de manuais metodológicos.

A pesquisa realizada pela Internet utilizou como principal fonte de busca as bases de artigos científicos *ScienceDirect, Scopus, Web of Science, Wiley Onlline Libery, Scientific Eletronic Library OnLine* (SCIELO), *Springer, PubMed* e ainda as bases de dados *Researchgate, Protein Data Bank, National Center for Biotechnology Information* (NCBI), Universal Protein (UniProt), The Comprehensive Enzyme Information System (BRENDA). As principais palavras-chave e equações de busca foram: *enzyme AND oil crude OR TPH OR petroderivative*, *oil AND biocatalysis OR enzyme*, *PAH-degrader*, *petroleum degradation AND enzymes, enzymes AND petroleum, enzymatic technology*, *enzymatic degradation* e outros. Os dados encontrados sobre o tema abordado foram utilizados para a análise das amostras em laboratório, para o embasamento do referencial teórico para a produção de artigos científicos provenientes da pesquisa em questão.

3.2 CONSÓRCIO MICROBIANO HIDROCARBONOCLÁSTICO

Os microrganismos investigados neste experimento fazem parte de um consórcio formado por dez bactérias e 24 fungos filamentosos nomeados sob os códigos descritos no Quadro 1 e protegidos pela patente de Lima et al. (2021), cujo número de depósito no Instituto Nacional da Propriedade Industrial (INPI) é BR 10 2021 002341 4. Essas cepas são oriundas de amostras de sedimento de manguezal da baía de Todos os Santos e de petróleo da Bacia do Recôncavo Baiano e foram testadas no trabalho de Dantas (2016), e apresentaram características promissoras a degradação de hidrocarbonetos do petróleo, conforme visto em Lima e colaboradores (2018).

Quadro 1 – Cepas que compõem o consórcio microbiano da patente n° BR 10 2021 002341 4 no INPI

Tipo	Código	Referências
Fungos	R11, R16, R26, R27, R28, R30, R31, S36, S38, S39, S40, S41, S45, S52, S53, A79, A80, A83, A84, N82, N89, N96, N101 e N102	Lima, 2016; Lima et al., 2017
Bactérias	RB1, RA2, RC5, RC6, PD4, PD5, PD6, PD7, PD8 e OH4	Dantas, 2016; Dantas et al., 2018

Fonte: Autora, 2022.

3.3 REATIVAÇÃO E MANUTENÇÃO DOS MICRORGANISMOS

As cepas encontram-se acondicionadas em preservação específica no cepário da Unidade de Geomicrobiologia do LEPETRO. Os fungos estavam sob refrigeração a 4 °C em preservação por água destilada estéril descrita por Castellani (1939), já as bactérias estavam em meio nutriente ágar com glicerol a 10 % sob refrigeração a - 20 °C.

As culturas bacterianas foram reativadas através da técnica de semeadura por estrias múltiplas em placa de Petri contendo 28 $g.L^{-1}$ do meio ágar Nutriente (Kasvi®). O material preservado foi inoculado na superfície do ágar com auxílio da alça de platina, formando quadrantes de estrias em zigue-zague. Em seguida as placas foram invertidas e incubadas em câmara de germinação TE402 (Tecnal) por 24 h a 30 °C. Para as cepas fúngicas foi adotado o método de repique (Figura 2) onde cubos de micélio (0,5 cm Ø) foram inoculadas em placa de Petri contendo 65 $g.L^{-1}$ do ágar Sabouraud Dextrose (Fluka® Analytical) adicionados a 0,1 $g.L^{-1}$ Cloranfenicol (Vetec®) e consecutivamente incubadas por 7 dias a 30 °C. As cepas fúngicas mais antigas que obtiveram resistência para reativação foram cultivadas nos meios confeccionados por Extrato de levedura (Sigma-Aldrich®), ágar Peptona + (Sigma-Aldrich®) e Dextrose Anidra (Impex) ou 3,27 $g.L^{-1}$ de caldo Bushnell-Haas (Difco™) acrescido de 1 % (v/v) de petróleo da bacia do Recôncavo Baiano e posteriormente incubada por 15 dias a 37 °C.

Figura 2 – Reativação dos microrganismos do consórcio que compõem a patente n° BR 10 2021 002341 4 no INPI. (a) Técnica de repique de fungos filamentosos. (b) Cepas de fungos reativadas após 7 dias de incubação

A **B**

Fonte: Autora, 2022.

A manutenção dos microrganismos foi realizada a cada quatro meses para garantir que o material estivesse sempre novo para utilização no experimento. O procedimento adotado na manutenção dos fungos consistiu em retirar 3 discos de micélio (0,5 cm Ø) das bordas das colônias inverter e transferir para o centro de novas placas de Petri com ágar Sabouraud Dextrose e para estirpe bacteriana o inóculo mais isolado foi raspado e estriado na superfície de outra placa de Petri contendo ágar Nutriente. Ambos foram incubados em câmara de germinação a 30 °C por 7 dias e 24h respectivamente, e então armazenadas sob refrigeração a 4 °C. A constituição dos meios de cultura utilizados para repique e reativação dos microrganismos do consórcio foram descritos no Quadro 2.

Quadro 2 – Composição e concentração dos meios de cultura utilizados para cultivo de fungos e bactérias

Meio de Cultura	**Componentes**	**Concentração (g. L^{-1})**
Nutriente Agar (NA) Kasvi®	Peptona	5,0
	Cloreto de sódio	5,0
	Extrato de levedura	2,0
	Extrato de carne	1,0
	Agarose	15,0
Sabouraud Dextrose (SDA) Fluka® Analytical	D+ glucose	40,0
	Peptona	10,0
	Agarose	15,0
Bushnell-Haas Broth (BH) Difico ™	Sulfato de magnésio	0,2
	Cloreto de cálcio	0,02
	Sulfato de monopotássio	1,0
	Fosfato de hidrogênio diamônio	1,0
	Nitrato de potássio	1,0
	Cloreto de ferro	0,05
Yest extract, Peptone e Dextrose (Y.P.D) Impex[Ltd], Sigma-Aldrich® e Impex[Ltd]	Extrato de levedura	10,0
	Peptona	20,0
	Dextrose	20,0
	Agarose	15,0

Fonte: Informações do fabricante, 2020.

3.4 EXTRAÇÃO E AMPLIFICAÇÃO DE DNA DOS MICRORGANISMOS

O método de extração de fungos ocorreu partir do kit de extração manual NORGEN Fungi/Yeast genomic DNA Isolation Kit (Norgen Biotek Corp), seguindo protocolo do fabricante (NORGEN, 2014) e submetido a banho ultrassônico (Elma Transsonic, TI-H10) a 130 khz por ± 8 minutos após etapa C do protocolo. A extração de DNA total das bactérias foi automatizada com o equipamento Maxwell® utilizando o kit Maxwell® 16 simply-RNA cell (Promega) na configuração LEV (*low elution* volume), seguindo as informações do fabricante (PROMEGA, 2014).

O DNA dos fungos foi submetido à amplificação por PCR (Reação em Cadeia de Polimerase) dos fragmentos de DNA da região ITS utilizando os *primers* ITS4 (R) e ITS5 (F) descritos, no Quadro 3. O preparo do *master mix* consistiu em 9,0 µL de TopTaq Mastermix® (Quiagem), 1,3 µL de água ultrapura (LGC Technology®), 0,2 µL de Primer ITS4 a 10 pmol, 0,2 µL de ITS5 a 10 pmol, 3,0 µL do aditivo Trealose, BSA e Tween-20 (TBT), 0,3 µL de Dimetilsulfóxido (DMSO) (Merck) e 1 µL (40 ng) de DNA total, compondo a reação em 15,0 µL. As condições de amplificação regida em termociclador (SureCycler 8800 Thermal Cycler - Agilent) foram provenientes do protocolo do Laboratório de Micologia (LAMIC) da UEFS, onde o passo inicial foi de um ciclo de desnaturação a 94 °C com duração de 5 minutos, seguido de 35 ciclos. Os ciclos foram compostos pelas etapas de desnaturação a 94 °C por 45 segundos, anelamento por 45 segundos a 55 °C e extensão por 1 minuto a 72 °C e um ciclo de extensão final a 72 °C por 7 minutos.

A amplificação do DNA das bactérias foi realizada a partir do gene 16S com os *primers* universais 4V(F) e 5V(R) descritos no Quadro 3. O preparo da solução *Master Mix* seguiu o mesmo protocolo utilizado nas amostras de fungos substituindo os primers. A PCR foi conduzida em termociclador (SureCycler 8800 Thermal Cycler - Agilent) utilizando a programação de um ciclo de desnaturação a 95 °C com duração de 10 minutos, seguido de 30 ciclos. Os ciclos foram compostos pelas etapas de desnaturação a 95 °C por 40 segundos, seguida de anelamento por 40 segundos a 55 °C e extensão por 1 minuto a 72 °C e um ciclo de extensão final a 72 °C por 10 minutos.

Quadro 3 - Primers utilizados para amplificação e sequenciamento dos genes ITS e 16S das amostras de DNA

Locus	Nome	Direção	Sequência (5′-3 ′)	Referência
Internal Transcribed Spacer (ITS)	ITS5	Forward	GGAAGTAAAAGTCGTAACAAGG	White et al.,1990
	ITS4	Reverse	TCCTCCGCTTATTGATATGC	White et al.,1990
16S	V4	Forward	CCTACGGGRSGCAGCAG	Wang; Qian, 2009
	V5	Reverse	CTTGTGCGGGCCCCCGTCAATTC	Wang; Qian, 2009

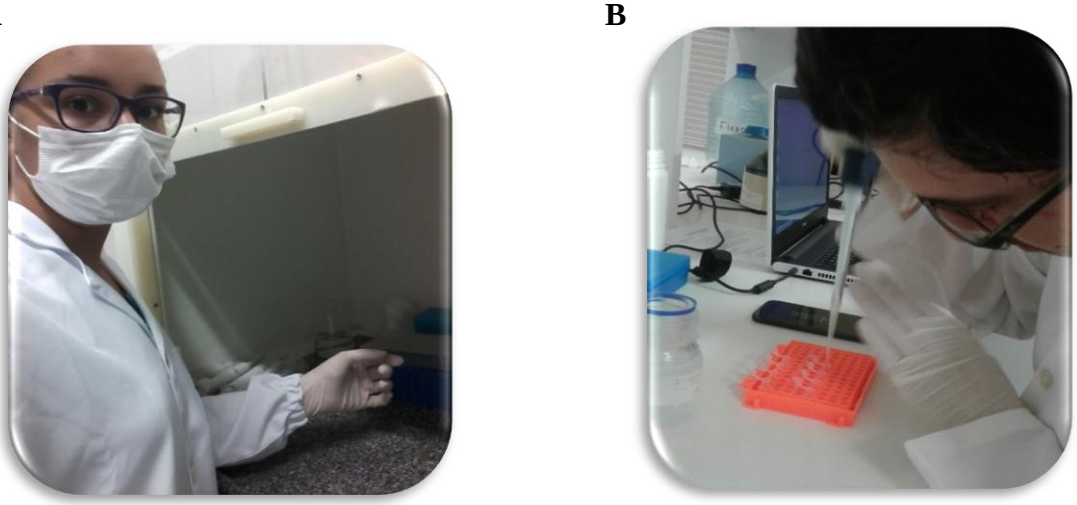
Fonte: Autora, 2022.

Após extração e amplificação dos nucleotídeos a quantidade e pureza do DNA total foi realizada por dois métodos. Primeiramente, utilizou-se o Quantus™ Fluorometer, onde 1 µL da amostra foi misturado em *vórtex* K40-1028 (Kasvi®) por 5 segundos a 199 µL do reagente ONEDNA e em seguida centrifugado por 5 segundos e incubado no escuro por 5 minutos antes da leitura. Em contrapartida, as amostras também foram quantificadas em espectrofotômetro NanoDrop ONE® (Thermofisher) utilizando 2 µL da amostra para leitura. Ambos os resultados

foram expressos em ng.µL. Adicionalmente a esse método, as amostras foram verificadas quanto a qualidade por eletroforese em gel de agarose e posterior visualização sob luz UV pelo transiluminador LPix (Loccus Biotecnologia) e as imagens capturadas e editadas pelo *software* L-Pix Image. O protocolo adotado na eletroforese foi 5,0 µL de corante Azul de Bromofenol a 0,4 % (Vetec Química®), 4,0 µL da amostra e 1,0 µL de GelRed® (Biotium) submetida a 100 Voltz por 40 min em gel de agarose (Life Tecnologia®) a 2 % (v/v) em tampão TBE (± pH 8,0). Na corrida de eletroforese foi acrescido um marcador de peso molecular de 100 pares de base (pb) Ladder (GE Healthcare®) para verificação do tamanho dos fragmentos.

Figura 3 – Extração e amplificação de DNA de microrganismos do consórcio da patente n° BR 10 2021 002341 4 no INPI. (a) Extração de DNA de fungos filamentosos pelo kit Norgen. (b) Preparo de Master Mix para amplificação de nucleotídeos em termociclador

A **B**



Fonte: Autora, 2022.

3.5 SEQUENCIAMENTO DE DNA E IDENTIFICAÇÃO DE MICRORGANISMOS

A purificação das amostras de DNA amplificado parcialmente ocorreu através do protocolo de precipitação de DNA utilizando Polietileno Glicol 8000 (PEG) (Sigma-Aldrich®) a 20 %. Adicionou -se igual volume de PEG a 20 % ao volume de amostra e em seguida foi incubado por 15 minutos a 37 °C e centrifugado por 15 minutos a 12000 rpm. Em sequência foi retirado o sobrenadante, adicionado 125 µL de etanol (Merck KGaA®) a 80 % gelado e a solução centrifugada por 5 minutos a 12000 rpm. Então o sobrenadante foi descartado e repetiu-se o passo anterior. As amostras foram secas em temperatura ambiente e ressuspensas com 10 µL de água ultrapura (LGC Technology®).

Em sequência as amostras foram submetidas a reação de sequenciamento pelo método direto nas direções de ida e volta (*forward* e *reverse*). A amostras foram preparadas em solução composta por 50 ng de produto de PCR purificado, 1,0 μL de tampão de sequenciamento (Save Money), 0,5 μL de BigDye™ Terminator v3.1 (Applied Biosystems®), 0,25 μL dos primers acima citados (3 pmol), completando para 10 μL de solução com água ultra pura (LGC Technology®). A reação de sequenciamento foi realizada com 35 ciclos compostos por uma etapa a 96 °C por 15 segundos, uma etapa a 52 °C por 10 segundos e uma etapa de polimerização a 60 °C por 4 minutos. Em seguida, adicionou-se 80 μL de isopropanol (Merck®) e incubadas por 15 minutos a temperatura ambiente. O DNA foi precipitado por meio da centrifugação a 4.000 rpm, por 25 minutos a 4 °C e então o sobrenadante foi descartado e as amostras lavadas duas vezes, com 150 μL de etanol (Qhemis) a 70 % seguido de centrifugação a 4.000 rpm por 15 minutos a 4 °C. As amostras foram secas em termociclador a 50 °C durante 15 minutos, ressuspendidas com 10 μL de formamida (Merck®) e desnaturadas por 5 minutos a 95 °C em bloco aquecedor (AccuBlock™, Labnet). Por fim, os produtos da reação foram submetidos ao sequenciamento pelo método de Sanger através do sequenciador automático 3500 DNA Analyzer (Applied Biosystems®) conforme metodologia sugerida pelo fabricante.

As sequências *forward* e *reverse* obtidas foram unidas para um comprimento comum e editadas a fim de remover resíduos de reagentes através do *Softwere* BioEdit versão 7.0.5 (HALL, 1999) e alinhadas utilizando o programa MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms versão 10.2.5 (KUMAR et al., 2018). Após edição, as sequências de nucleotídeos foram comparadas com as sequências conhecidas na base de dados do GenBank™ utilizando o programa BLAST (ALTSCHUL et al., 1990) do National Center for Biotechnology Information (NCBI), disponível em: <http://www.ncbi.nlm.nih.gov/BLAST/> para realizar a identificação das cepas e posterior registro na base.

A distância evolutiva das espécies foi analisada através construção de árvore filogenética pelo método de Neighbor-Joining (NJ) utilizando o programa MEGA X (versão 10.2.5). O cladograma foi montado com matriz de caracteres com todos os táxons, utilizando o modelo K2P e aplicando-se 1000 réplicas de *bootstrap* (FELSENSTEIN, 1985), para obter o suporte dos agrupamentos. Foram selecionados para compor o grupo externo do cladograma de fungos e bactérias a espécie *Fusarium solani* registrada no NCBI sob o código NR_163531.1, e a espécie *Corynebacterium propinquum* depositada no NCBI sob código HE962219, respectivamente.

3.6 DELINEAMENTO EXPERIMENTAL

O experimento foi constituído de dois tratamentos: Consórcio cultivado na presença do petróleo (CO) e consórcio cultivado em glicerol (CG) acompanhado de seus respectivos controles negativos. O teste foi conduzido com cinco réplicas biológicas, três réplicas de controle negativo e dois brancos para os tempos amostrais 0 dia e 7 dias de incubação, totalizando 32 amostras.

A composição das unidades experimentais e respectivos volumes foram descritos conforme delineamento experimental do Quadro 4.

Quadro 4 – Delineamento experimental para identificação de enzimas e degradação biótica e abiótica do petróleo

Quantidade	Unidade experimental	Composição	Volume (mL)
5	CO	Meio de cultura Bushnell-Haas Broth	100
		Solução matriz do consórcio	10
		Petróleo do Recôncavo Baiano	1
3	BO	Meio de cultura Bushnell-Haas Broth	100
		Petróleo do Recôncavo Baiano	1
5	CG	Meio de cultura Bushnell-Haas Broth	100
		Solução matriz do consórcio	10
		Glicerol	1
3	BG	Meio de cultura Bushnell-Haas Broth	100
		Glicerol	1
2	BR	Meio de cultura Bushnell-Haas Broth	100

Fonte: Autora, 2022.

A montagem do experimento foi conduzida com a adição de alíquota de 10 mL da solução matriz do consórcio microbiano padronizado a 12,089 x 10^3 UFC.mL e transferidas assepticamente para frascos Erlenmeyers de 250 mL contendo 100 mL de caldo mineral Bushnell-Haas (Difco™) e 1 mL (± 0,8 g) de petróleo. Vale ressaltar que o óleo utilizado nesse experimento, corresponde ao óleo da Bacia de Recôncavo Baiano (APÊNDICE 16 e 17), cedido pela operadora independente de exploração e produção de petróleo e gás *on shore* Petrorecôncavo ao LEPETRO/IGEO/UFBA para a realização de pesquisas acadêmicas no setor. Desse petróleo foram retiradas duas amostras *background* para caracterização em cromatógrafo equipado com um detector de ionização de chamas. O caldo de sais marinhos Bushnell-Haas foi inicialmente descrito por Bushnell e Haas (1941), para a avaliação da capacidade de microrganismos em decompor hidrocarbonetos, cuja fórmula não apresenta carbono. Além disso, é recomendado para o exame microbiológico de combustíveis pelo Comitê de Deterioração Microbiológica de Combustíveis da Sociedade de Microbiologia Industrial (SIM) (ALLRED et al., 1963). Como fonte alternativa de carbono utilizamos 1 mL de glicerol (Invitrogen) e o controle foi constituído de caldo e óleo ou caldo e glicerol, enquanto os brancos foram montados apenas com o caldo

mineral. Os controles e brancos foram submetidos a teste de esterilidade com o objetivo de garantir que a reação esteja ocorrendo somente na presença dos microrganismos adicionados. O teste de esterilidade consistiu na contagem de unidades formadoras de colônias (UFC) por meio da técnica de diluição seriada segundo Romeiro (2001), e Gerba e Pepper (2004), para bactérias e fungos respectivamente.

As reações foram realizadas em incubadora *shaker* orbital (Lab Companion) sob aquecimento a 35 °C e agitação a 180 rpm durante 7 dias. Para evitar a evaporação das amostras e volatilização do óleo, os frascos de Erlenmeyer foram fechados com papel alumínio e vedados com papel filme. Além disso, a incubadora foi forrada com papel pardo para evitar a fotodegradação. As vidrarias utilizadas, bem como o meio de cultura foram previamente descontaminados conforme descrito no tópico 4.18 Remoção de contaminantes e descarte de resíduos.

No intervalo de 0 e 7 dias foram coletadas alíquotas (Figura 4) para avaliar a concentração de hidrocarbonetos totais do petróleo (HTP), hidrocarbonetos policíclicos aromáticos (HPA), crescimento de microrganismos, atividade enzimática, proteínas totais e identificação de proteínas. No período estipulado de coleta as amostras foram homogeneizadas e armazenadas em freezer a -20 °C para análise orgânica e para as análises microbiológicas em freezer a -80 °C até que pudessem ser analisadas, com exceção da atividade enzimática que foi mensurada no mesmo dia.

Figura 4 – Delineamento experimental para identificação de proteínas do consórcio e avaliação da degradação de hidrocarbonetos do petróleo. (a) Unidades experimentais em incubadora *shaker*. (b)Coleta de amostras para realização das análises geoquímicas e microbiológicas

A **B**

Fonte: Autora, 2022.

3.7 CULTIVO DO PRÉ-INÓCULO E SOLUÇÃO MATRIZ

Inicialmente foi preparado um pré-inóculo para cada cepa fúngica e bacteriana e posteriormente uma solução matriz com todos os microrganismos do consórcio (Figura 5). A solução microbiana individual foi constituída de 10 mL NaCl a 0,9 % (Synth), 0,3 % de Tween 80 (Synth) e 10 discos (1 cm Ø) de agarose cultivados com a cepa e recortado com auxílio da ponteira de 1000 µL. A amostra foi homogeneizada com a adição de duas esferas de vidro (1,5 cm Ø) e submetido a vigorosa agitação a 25 rpm por aproximadamente 2 minutos ou até dissolver os discos.

Posteriormente 1 mL de cada pré- inóculo foram transferidos para frascos Erlenmeyers de 500 mL contendo 250 mL de meio BH (Difco™) suplementado com 1 % (v/v) de glicerol (Invitrogen). Os meios foram incubados em mesa agitadora orbital Tecnal, modelo TE-420 a 180 rpm durante 7 dias a 30 C° ± 0,2 com quatro repetições independentes. Para controle do experimento uma amostra foi incubada com o mesmo meio mineral sem a adição dos microrganismos e submetida sob as mesmas condições de cultivo. Em seguida, o volume total das quadruplicatas da solução do consórcio foi homogeneizado entre si a fim de reduzir a diferença de crescimento biológico entre as réplicas e formar uma solução matriz única. Assim, a solução matriz final do consórcio foi armazenada a 4 °C até utilização nos experimentos subsequentes.

Figura 5 – Preparo do pré-inóculo e solução matriz do consórcio microbiano hidrocarbonoclástico. (a) Homogeneização do pré-inóculo das cepas individuais do consórcio. (b) Solução matriz incubada em mesa agitadora

A **B**

Fonte: Autora, 2022.

3.8 QUANTIFICAÇÃO DE MICRORGANISMOS

O pré-inóculo e a solução matriz do consórcio foram quantificadas através de três métodos a fim de aumentar as chances de reprodutividade da solução. O primeiro método foi através da leitura em espectrofotômetro, modelo LMR-96 (Loccus), onde 200 µL da solução foi adicionada em microplaca de 96 poços e posteriormente verificado a absorbância utilizando os filtros 492 nm e 600 nm após agitação automatizada por 40 segundos. O segundo método foi realizado fazendo-se a contagem de células viáveis através da visualização em câmara de Neubauer segundo as orientações modificadas de Nucarini et al. (2004). O preparo da amostra ocorreu por meio da diluição de 250 µL de cada cepa microbiana em 250 µL de solução salina a 0,9 % e 500 µL de corante Azul de Metileno (Merck®) a 0,01 %. Posteriormente a solução foi homogeneizada em *vortex* por 5 segundos e uma alíquota de 10 µL foi retirada dessa solução e inserida na câmara de Neubauer (NewOptics) de 0,0025 mm^2 e 0,100 mm de profundidade. Após inserção em câmera aguardou-se entre 1 a 2 minutos para que as células solubilizassem e iniciasse a contagem em microscópio óptico em lente de 40 X para fungos e lente de 100 X para bactérias. Nessa ocasião, somente células não coradas de azul ou azul claro eram contabilizadas como células viáveis e considerou-se também as células que estivessem as linhas superior e as linhas à esquerda. Os quadrantes escolhidos para quantificação foram os 5 referentes ao compartimento central e posteriormente submetido a cálculo de diluição correspondente (Equação 1). Os resultados referentes a essa quantificação foram inseridos no APÊNDICE 14.

Equação 1:

$$\frac{Células}{mL} = \frac{(Q1 + Q2 + Q3 + Q4 + Q5)}{5} \; x \; 2{,}5 \; x \; 10^5$$

Onde:

Q = Quantidade total de células contadas em cada quadrante

5 = Quantidade de campos contados

2,5 x 10^5 = Fator de diluição (relacionado ao volume e profundidade da câmara)

O terceiro método de quantificação foi através da diluição seriada, o qual também foi aplicado no experimento de biocatálise/proteômica (Figura 6). A quantificação de bactérias foi realizada conforme método de diluição em série por micro-gota / *spread plate* (ROMEIRO, 2001). Inicialmente, em capela de fluxo laminar, foi obtida uma suspensão contendo 10 mL de

amostra e 90 mL de solução salina (0,9 %) estéril. A partir dessa suspensão, foram realizadas diluições sucessivas até diluição 10^{-8} com alíquotas de 100 µL em microtubos que continham 900 µL de solução salina (0,9 %) estéril. Em seguida, realizou-se o plaqueamento com o auxílio de micropipeta, adicionando-se 4 gotas de 10 µL na superfície de placas de Petri esterilizadas com meio de cultura ágar nutriente (Kasvi®). Após completa absorção das gotas no meio de cultura, as placas foram incubadas a 30 °C ± 1 °C por 24 horas. Após o período de incubação, foi feita a contagem manual das colônias de bactérias e os resultados foram expressos em UFC.mL conforme Equação 2.

Para a contagem das cepas fúngicas foi utilizado o método de diluição seriada/*placa pour* (GERBA; PEPPER, 2004). A suspensão inicial foi realizada conforme descrito anteriormente. Em tubos de ensaio foram adicionados 9 mL de solução salina (0,9 %) estéril e a partir de então efetuou-se a série de diluições, até diluição 10^{-5} com alíquotas de 1 mL da suspensão inicial. Em placas de Petri vazias e estéreis foram adicionadas alíquotas de 1 mL correspondente a cada diluição e posteriormente o meio de cultura ágar Sabouraud Dextrose (Fluka® Analytical) foi vertido (< 50 °C) sobre as alíquotas. As placas foram invertidas e incubados a 30 °C ± 1 °C por 7 dias. Posteriormente foi realizada a contagem manual das colônias de fungos e os resultados foram expressos em UFC.mL^{-1} conforme Equação 2, excluindo-se a primeira diluição do cálculo e a divisão por número viável de diluições.

Equação 2:

$$UFC = \frac{\sum\left(\frac{N°colônias}{4}\right) x\ f.d}{N°d.v}$$

Onde:

f.d = Fator de diluição

Σ = Somatório de todas as diluições

N d.v = Número de diluições viáveis

4 = Número de alíquotas da mesma suspensão

Figura 6 – Quantificação de fungos e bactérias através do método de diluição seriada. (a) Diluição em série de amostras bacterianas até diluição 10^{-8}. (b) Diluição em serie de amostras fúngicas após 7 dias de incubação

A **B**

Fonte: Autora, 2022.

3.9 EXTRAÇÃO DE PROTEÍNAS

As proteínas foram extraídas a partir do método modificado descrito por Isaacson et al. (2006). Inicialmente as amostras foram submetidas a um pré-tratamento que consiste na centrifugação para reduzir a interferência do petróleo bruto nas análises subsequentes. Para tal, 100 mL da amostra foi centrifugada a 4500 rpm por 15 minutos a 10 °C (Figura 7) e o precipitado ressuspendido com 2 mL de tampão de extração constituído de Sacarose (Exôdo) a 0,7 M, Tris-base (Ludwig Biotec) a 0,5 M, cloreto de potássio (Merck®) a 0,1 M, ácido clorídrico (Química Nova) a 0,09 %, ácido etilenodiamino tetra-acético (EDTA) (Fisher Bioreagents) a 0,05 M e 40 µL Diotiotreitol (DTT) (LGC Biotecnologia®) a 40 mM. A quebra celular foi realizada sob agitação de 30 rpm com esferas de vidro (3 mm) por 3 vezes durante 5 minutos com intervalo de 1 minuto no gelo. Em seguida foi centrifugada em centrífuga refrigerada Universal modelo 320R (Hettich) durante 15 minutos a 14000 rpm a 10 °C e o precipitado pesado.

Após centrifugação, o sobrenadante foi transferido para microtubos e mesclado com dois volumes de acetato de amônio gelado e mantido sob incubação a -80 °C durante 2 horas. Após período de precipitação, o *pellet* foi adquirido por meio de centrifugação durante 15 minutos a 14000 rpm. Por conseguinte, o *pellet* foi lavado 3 vezes com 500 µL de acetato de amônio gelado e depois 3 vezes com 500 µL de acetona (Synth) a 80 %. A amostra foi seca à temperatura ambiente durante 20 minutos, ressuspendida em 200 µL de tampão de solubilização composto

por bicarbonato de amônio (Sigma-Aldrich®) a 50 mM, pH 8,0 e armazenada a -20 °C para análises posteriores.

Após quantificação de proteínas e visualização das bandas em gel, o material foi desidratado durante 1 hora e 30 minutos com auxílio de um concentrador rotacional a vácuo, modelo RVC 2-18 CDPlus (Christ), não refrigerado para posterior análise em cromatografia líquida com espectrômetro de massas (LC-MS).

Figura 7 – Extração de proteínas a partir de consórcio microbiano hidrocarbonoclástico. (a) Alíquota da amostra CG em 7 dias. (b) Decantação do substrato por centrifugação refrigerada

A **B**

Fonte: Autora, 2022.

3.10 DETERMINAÇÃO DA CONCENTRAÇÃO DE PROTEÍNAS TOTAIS

A quantificação de proteínas totais foi de acordo com o método de Bradford (1976) conforme demonstrado na Figura 8. Esse método, apesar de antigo, ainda é muito utilizado em várias aplicações sendo considerado um dos mais sensíveis com limite de detecção de 2×10^{-5} e rápidos com reação em 2 minutos (WILSON; WALKER, 1995). O princípio do método está relacionado com interação entre o corante *Coomassie brilliant blue* e os aminoácidos de cadeias laterais básicas ou aromáticas, onde a cor do corante muda de marrom para azul quão maior for à concentração da amostra (BRADFORD, 1976). O processo de quantificação ocorreu através da leitura das amostras em espectrofotômetro, modelo LMR 96-4 (Loccus) em microplaca a absorbância de 595 nm. Para quantificar a concentração de proteínas na amostra foi construída uma curva analítica de calibração utilizando com padrão a Albumina Sérica Bovina (BSA) nas concentrações 0 a 200 $\mu g.mL^{-1}$. Inicialmente foi preparada uma solução matriz de BSA (Sigma-Aldrich®) a 500 $\mu g.mL^{-1}$ e então diluída em água ultrapura (LGC Technologia®) formando 12

pontos conforme descrito no Quadro 5. O ensaio foi realizado em placa tipo ELISA de 96 poços com capacidade de volume final de 200 µL cada. Em cada poço foi adicionado 5 µL das alíquotas dos padrões homogeneizados em 195 µL da solução corante Azul de Coomassie EZBlue TM (Sigma-Aldrich®) e incubado por 15 minutos no escuro. O mesmo procedimento foi realizado com 5 µL da amostra para 195 µL do corante Azul de Coomassie EZBlue TM (Sigma-Aldrich®). O coeficiente de correlação linear adotado para construção da curva foi ≥ 0,98 e os valores da concentração das proteínas foram expressos em $\mu g.mL^{-1}$.

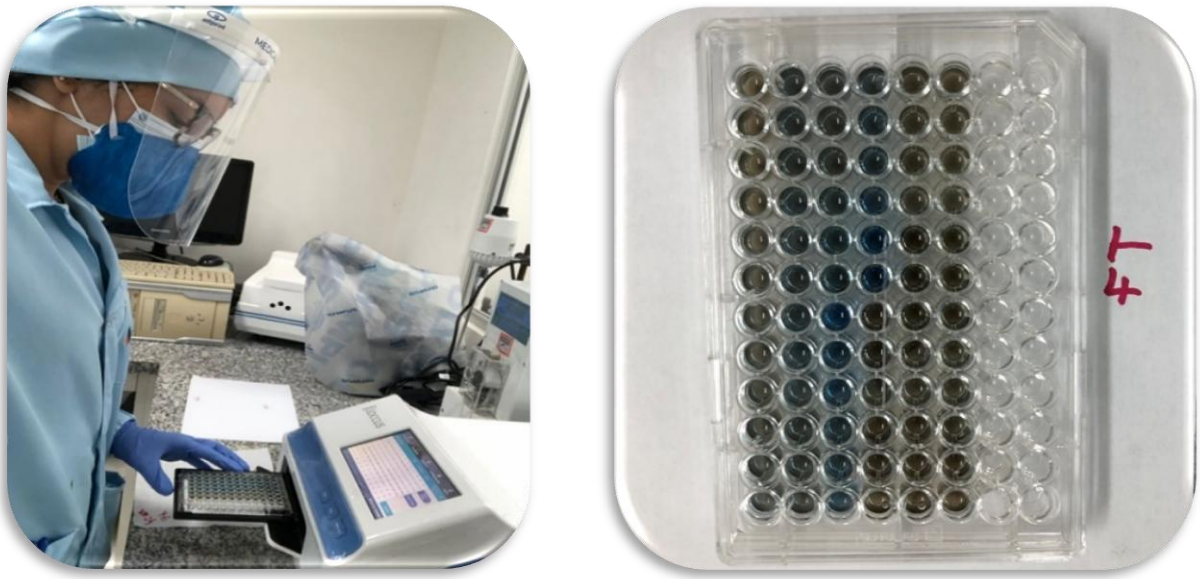
Quadro 5 – Concentração dos pontos da curva de calibração de proteínas utilizando o padrão BSA

Tubo	Concentração ($\mu g.mL^{-1}$)	Volume de água (µL)	Volume do padrão BSA (µL)	Volume final (µL)
1	0	200	0	200
2	5	198	2	200
3	15	194	6	200
4	30	188	12	200
5	45	182	18	200
6	60	176	24	200
7	75	170	30	200
8	90	164	36	200
9	120	152	48	200
10	150	140	60	200
11	170	132	68	200
12	200	120	80	200

Fonte: Autora, 2022.

Figura 8 – Quantificação de proteínas através do método de Bradford. (a) Leitura das amostras em epectrofotômetro de placas multipoços. (b) Amostras coradas com o corante Azul de Coomassie

A **B**



Fonte: Autora, 2022.

3.11 VISUALIZAÇÃO DE PROTEÍNAS POR ELETROFORESE EM GEL SDS-PAGE

O extrato protéico foi submetido ao método SDS-PAGE (LAEMMLI, 1970) em gel de acrilamida. Este método é baseado na desnaturação de proteínas utilizando dodecil sulfato de sódio (SDS) com a finalidade de monitorar a pureza, estimar quantidade e peso molecular das amostras (BRUNELLE; GREEN, 2014). As soluções para preparo dos géis e soluções tampões foram confeccionados conforme descrito no Quadro 6, onde adotou-se 12 % (v/v) de acrilamida para camada do gel de separação e 5 % para gel de empilhamento montados em um sistema de gel vertical de 17,5 cm x 17 cm. O protocolo adotado foi adição de 10 µL de amostra homogeneizada em tampão de amostra previamente aquecidas a 90 °C por 5 minutos e 5,0 µL do padrão Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific). A eletroforese foi executada a 150 Voltz a 400 mA 10 W durante ± 1:30 minutos e 180 Voltz por 4 h em tampão de corrida (pH 8,3 ± 0,1). Para visualização das bandas o gel foi submetido ao método de coloração com nitrato de prata, método de grande sensibilidade para detecção de proteínas podendo ser até 100 vezes mais sensível do que a coloração com Azul de Coomassie (MERRIL,1990) (Figura 9). O gel ficou submerso durante 7 minutos em 100 mL de solução fixadora compostas por ácido acético (Merck®), álcool etílico a 96 % (Qhemis) e 1 mL de nitrato de prata a 20 % (FMaia), sendo o último adicionado à solução somente na imersão do gel. Posteriormente o gel foi lavado três vezes com água destilada e então submerso em 100 mL de solução reveladora composta por hidróxido de sódio a 22,5 % (Química Moderna) e 200 uL de formaldeído (Vetec) a 36 % aplicado no momento de uso. A revelação ocorreu sob agitação manual por aproximadamente 10 minutos até completa visualização das bandas e a reação interrompida com álcool e sucessivas lavagens em água destilada. Posteriormente, o gel foi incubado *overnight* em água destilada e fotografado para a visualização dos spots. Em seguida foi envolvido com papel celofane transparente e seco a temperatura ambiente (APÊNDICE 24).

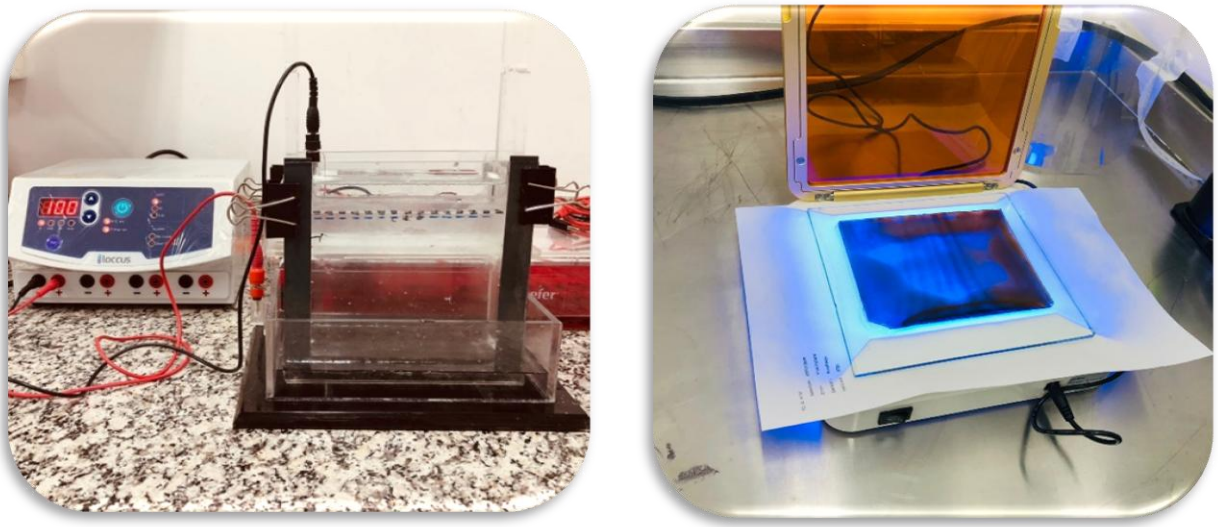
Quadro 6 - Composição e concentração das soluções para construção e revelação de gel pelo método SDS-PAGE

Solução	Quantidade	Componente	Concentração
Gel de separação	7,5 mL	Tampão Tris-HCl, SDS (Ludwig Biotec)	1 M (pH 8,8), 0,4%
	13 mL	Bis-acrilamida (Bio-Rad)	30%
	0,40 mL	Persulfato de amônio (APS) (Sigma-Aldrich)	20%
	0,04 mL	TEMED (Sigma-Aldrich)	NA
	100 mL	Água destilada	NA
Gel de empilhamento	1,85 mL	Tampão Tris-HCl, SDS	1,5 M (pH 6,8), 0,4%
	1 mL	Bis-acrilamida (Bio-Rad)	30%
	0,25 mL	Persulfato de amônio (APS) (Sigma-Aldrich)	20%
	0,02 mL	TEMED (Sigma-Aldrich)	NA
	4,5 mL	Água destilada	NA
Tampão de corrida (5x)	15,1 g	Tris-HCl (Ludwig Biotec)	0,025 M (pH 8,3)
	72,1 g	Ácido aminoacético (Synth)	0,192 M
	5 g	SDS (LGC-Biotecnologia)	0,1%
	5 L	Água destilada	NA
Tampão de amostra (5x)	1 mL	Solução Tris-HCl (Ludwig Biotec)	0,5 M pH 6,8
	1,6 mL	SDS (LGC-Biotecnologia)	10%
	0,8 mL	Glicerol (Synth)	NA
	0,4 mL	2-β-Mercaptoetanol (Sigma-Aldrich)	NA
	0,2 mL	Azul de Bromofenol (Sigma-Aldrich)	NA
	4 mL	Água destilada	NA
Solução fixadora	7 mL	Ácido acético (Merck)	NA
	160 mL	Álcool etílico absoluto (Qhemis)	96 %
	833 mL	Água destilada	NA
Corante	1 mL	Nitrato de prata (FMaia)	20%
Solução reveladora	22,5 g	Hidróxido de sódio (Química Moderna)	NA
	1 L	Água destilada	NA

*NA, Não se aplica
Fonte: Autora, 2022.

Figura 9 – Visualização de amostras protéicas em gel de bisacrilamida a 12 %. (a) Sistema de eletroforese de gel SDS-PAGE. (b) Fotodocumentação das amostras proteicas coradas com nitrato de prata

A **B**



Fonte: Autora, 2022.

3.12 DETERMINAÇÃO DA ATIVIDADE DE ENZIMÁTICA

As análises para determinação das atividades enzimáticas de lacase, manganês peroxidase, lignina peroxidase, alcano oxigenase e catecol 2,3 dioxigenase foram realizadas em espectrofotômetro de microplacas, modelo LMR 96-4 (Loccus) através da leitura dos sobrenadantes do experimento conforme descrito a seguir e resumido no Quadro 7 e Figura 10**.**

A atividade enzimática da lacase (Lac) foi determinada através da oxidação do substrato à base de siringaldazina e posterior formação de quinona (SZKLARZ et al., 1989). O teste foi realizado em microtubos, onde foram adicionados 100 µL do extrato enzimático, 80 µL de tampão citratofosfato a 0,05 M (pH 7) e 20 µL de solução de siringaldazina (Sigma-Aldrick) a 0,1 % (em etanol). A reação foi avaliada através da leitura em espectrofotômetro no comprimento de onda 525 nm, nos tempos 0 minutos e após 10 minutos de reação. O coeficiente de absorção molar considerado para cálculo da equação foi de 65000 $L.M^{-1}.cm^{-1}$.

A atividade enzimática da lignina peroxidases (LiP) foi determinada a partir da oxidação do álcool veratrílico (ARORA; GILL, 2001). A reação ocorreu por meio da mistura de 40 µL extrato enzimático, 80 µL de tampão tartarato de sódio a 125 mM (pH 3,0), 40 µL de álcool veratrílico a 10 mM e por último a adição 40 µL de peróxido de hidrogênio a 2 mM para iniciar a reação. A formação do aldeído veratrílico foi avaliada mediante leituras em espectrofotômetro por absorbância de 310 nm, em 0 minutos e após 10 minutos de incubação. O coeficiente de absorção molar considerado para cálculo da equação foi de 9300 $L.M^{-1}.cm^{-1}$.

A atividade enzimática da enzima manganês peroxidase (MnP) foi determinada através da oxidação do vermelho de fenol (KUWAHARA et al., 1984). A reação ocorreu através da adição 100 µL do extrato enzimático, 10 µL sulfato de manganês a 2 mM, 10 µL de peróxido de hidrogênio em tampão succinato de sódio a 20 mM (pH 4,5), 20 µL de vermelho de fenol a 0,01 %, 20 µL de lactato de sódio a 0,25 M), 40 µL de albumina bovina a 0,5 %. A reação foi avaliada lendo-se a absorbância à 610 nm em 0 minutos e após 10 minutos. Foi adicionado 10 µL solução de hidróxido de sódio a 2,0 N para interromper a reação. O coeficiente de absorção molar considerado para cálculo da equação foi de 22000 $L.M^{-1}.cm^{-1}$.

A atividade de alcano hidroxilase (AH) foi mensurada pelo decréscimo de NADH (LEE et al., 1996). A reação consistiu na junção de 20 µL do extrato enzimático, 178 µL de tampão TRIS-HCl a 27 mM (pH 7,4), 20 µL de NADH (Sigma-Aldrick) a 1 mM, 2 µL de iso-octano (Merck®) a 1 % e 10 µL de CHAPS (Sigma-Aldrick) a 0,15 %. A atividade da enzima mencionada foi

medida em espectrofotômetro pela absorbância a 340 nm em 0 minutos e após 10 minutos. O coeficiente de absorção molar considerado para cálculo da equação foi de 6220 $L.M^{-1}.cm^{-1}$.

A atividade da catecol 2,3 dioxigenase (C2,3O) foi mensurada mediante oxidação de catecol e formação de hidroximucônico semialdeído (BAGGI et al., 1987). A reação foi realizada por meio da mistura de 67 µL de extrato enzimático, 66 µL de tampão fosfato a 50 mM (pH 7,0) e 67 µL de catecol (Sigma-Aldrick) a 0,3 mM. A reação foi avaliada lendo-se a absorbância à 375 nm em 0 minutos e após 10 minutos de incubação. O coeficiente de absorção molar considerado para cálculo da equação foi de 36000 $L.M^{-1}.cm^{-1}$.

As análises foram realizadas em triplicata e os resultados foram expressos em µmol min $litro^{-1}$ ($U.L^{-1}$), por meio da Equação 3 descrita abaixo e considerando os valores de coeficiente de absorção específico.

Equação 3:

$$(U.L) = (\Delta A)\ x \frac{10^6}{\varepsilon\ x\ R\ x\ T}$$

Onde:

ΔA = Diferença entre absorbância final e inicial
10^6 = Conversão de mols do ε em µmols
ε = Coeficiente de extinção (M^{-1} cm^{-1})
R = Quantidade de amostra (mL)
T = Tempo de reação (min.)

Figura 10 – Determinação de atividade enzimática de enzimas degradadoras de compostos do petróleo. (a)Preparo de soluções tampão para as reações de atividade enzimática. (b) Pipetagem das soluções para as reações de atividade enzimática

A **B**

Fonte: Autora, 2022.

Quadro 7 – Concentração e composição das soluções para determinação de atividade enzimática de enzimas degradadoras de compostos do petróleo

Enzima	Volume (µL)	Composição	Concentração	Observação
Alcano hidroxilase (340 nm)	20	Amostra	NA	NA
	148	Tampão TRIS-HCl (Ludwig Biotec)	20 mM	pH 7,4
	20	NADH(Sigma-Aldrich)	1 mM	NA
	2	Iso-octano (Merck®)	1%	em DMSO 80%
	10	CHAPS (Sigma-Aldrich)	0,15 %	NA
Lacase (525 nm)	100	Amostra	NA	NA
	80	Tampão citratofosfato	0,05 M	pH 7,0
	20	Siringaldazina (Sigma-Aldrich)	0,1 %	em Etanol
Manganês peroxidase (610 nm)	100	Amostra	NA	NA
	10	Sulfato de manganês (Sigma-Aldrich)	2 mM	NA
	10	Tampão succinato de sódio em peróxido de hidrogênio	20 mM;	pH 4,5
	20	Vermelho de fenol (Sigma-Aldrich)	0,01 %	em Etanol
	20	Lactato de sódio	0,25 M	NA
	40	Albumina bovina (Sigma-Aldrich)	0,5 %	NA
	10	Hidróxido de sódio (Química Moderna)	2,0 N	NA
Lignina peroxidases (310 nm)	40	Amostra	NA	NA
	40	Peróxido de hidrogénio (Merck®)	2 mM	NA
	40	Álcool veratrilico (Sigma-Aldrich)	10 mM	NA
	80	Tampão de tartarato de sódio	125 mM	pH 3,0
Catecol 2,3 dioxigenase (375 nm)	67	Amostra	NA	NA
	66	Tampão fosfato	50 mM	pH 7,0
	67	Catecol (Sigma-Aldrick)	0,3 mM.	em tampão

*NA, Não se aplica.
Fonte: Autora, 2022.

3.13 DIGESTÃO E DESSALINIZAÇÃO DO EXTRATO PROTÉICO

As análises protêomicas foram realizadas no Instituto de Biotecnologia (IBTEC), Universidade Estadual Paulista (UNESP) em Botucatu, São Paulo, com o auxílio dos pesquisadores Dr. Bruno C. Rossini e Dra Lucilene D. Santos. Inicialmente as amostras foram ressuspensas em tampão Bicarbonato de amônio a 50 mM (pH 7,8) e misturadas em vórtex por 2 minutos. Em seguida, as amostras foram centrifugadas a 14.000 x g por 15 minutos em temperatura ambiente. Os sobrenadantes foram transferidos para microtubos plásticos do tipo *lobind* e submetidos ao protocolo de digestão de proteínas em solução, conforme descrito por Sylvestre et al. (2018). Resumidamente, cada amostras (± 50 ug de proteínas totais) foram alquiladas na presença de 45 µM de iodoacetamida e, em seguida, submetidas à hidrólise de proteínas por incubação com a enzima tripsina (1 ng.µL) durante a noite a 37 °C. Extinguiu-se a reação adicionando 1 % (v/v) de ácido trifluoroacético e centrifugou-se a 15.000 × g por 2 minutos. As amostras foram então submetidas a cromatografia de fase reversa em colunas de dessalinização PeptideCleanup C_{18} Spin (Agilent Technologies) para separação dos peptídeos

trípiticos e secas em concentrador do tipo Speed vac. A quantificação dos peptídeos foi realizada através do método de fluorescência. Esse método é sensível baseado em fluorescência por meio de corante seletivo para proteínas minimizando efeitos da contaminação, cujos dados são gerados com base na relação entre os *Standards* usados na calibração (INVITROGEN, 2010). A florescência foi mensurada no equipamento Qubit® fluorometer (Invitrogen^{TM}). As amostras de proteína foram preparadas conforme protocolo do fabricante (INVITROGEN, 2010), onde 1 µL da amostra é misturada a 199 µL da solução de trabalho composta de Fluoróforo *Quant-iT reagente* diluído em tampão (1:200), incubado por 15 minutos no escuro e posteriormente realizada leitura no equipamento. Nessa etapa, as amostras foram solubilizadas em 100 uL de ácido fórmico (0,1 % AF) e 2 uL foram utilizados na quantificação por fluorescência.

3.14 SEQUENCIAMENTO DE PEPTÍDEOS POR LC-MS/MS

As análises de espectrometria de massa foram realizadas usando um sistema de nanocromatografia líquida Ultimate 3000 LC (Dionex) acoplado ao espectrômetro de massas Q-Exactive ™ Hybrid Quadrupole-Orbitrap ™ (Thermo Fisher Scientific). A ionização foi obtida usando fonte de íons Nanospray (PicoChip, Modelo 1PCH-550, 75 µm ReproSil Pur C_{18} 3 µm; New Objective, EUA) com pré-concentração em armadilha Acclaim PepMap 100 de 2 cm (75 µm ID, C18 3 µm; Thermo Fisher Científico). Aproximadamente 5 µg do produto da digestão tríptica foram injetados no sistema e então separados a partir da combinação das fases A e B. A fase móvel A consistia em 0,1 % de ácido fórmico em água e a fase móvel B de 0,1 % de ácido fórmico em acetonitrila sob fluxo constante de 300 $nL.min^{-1}$. O equipamento foi ajustado para o gradiente de 2 % a 40 % para fase móvel B ao longo de 2 horas, seguido por 10 minutos de lavagem da coluna em 80 % da fase móvel B e reequilíbrio da coluna por 10 minutos a 2 %. Os reagentes utilizados nessa etapa foram de alta pureza analítica, específico para LC/MS. O sequenciamento foi realizado por triplicata biológica e triplicata analítica.

3.15 IDENTIFICAÇÃO DE PROTEÍNAS

Os dados brutos foram submetidos à análise de bioinformática através do *software* PatternLab for proteomics versão 4.0.0.84 com resolução de 17.500, 50 ms de tempo de injeção, 1,2 m / z de janela de isolamento e tempo de exclusão dinâmica de 10 segundos (CARVALHO et al., 2016), disponível em:< http://www.patternlabforproteomics.org/>. Esse programa foi

utilizado para configurar os bancos de dados de sequência de aminoácidos, realizar correspondência de espectro de peptídeo e para filtrar e organizar os dados estatisticamente. A pesquisa MS/MS foi realizada utilizando a estratégia *shotgun* do tipo "bottom-up" (EMIDIO et al., 2015) com configurações que permitem seleção adequada das amostras alvo. Um método de ajuste não linear para a avaliação de confiança global dos dados proteômicos (TANG et al., 2008) foi aplicado ≤ 0,01 para taxa de descoberta falsa (FDR) (AGGARWAL; YADAV, 2016), ou seja, até 1 % do número total de espectro-peptídeo correspondentes (PSM) foi identificado e as proteínas agrupadas de acordo com o critério de máxima parcimônia, quando as sequências trípticas identificadas não foram suficientemente informativas para separação de dois eventos (ZHANG et al. 2007). E então os resultados foram analisados na UniProtKB que compreende os bancos de dados Swiss-Prot e TrEMBL, disponível em:< https://www.uniprot.org/ > para a identificação das proteínas e investigação das funções biológicas. Os espectros experimentais foram comparados com espectros de massas teóricos a partir da mescla de dois conjuntos de dados de sequências proteicas específica para os microrganismos que constituem o consórcio microbiano suplementada com proteínas específicas (APÊNDICE 15) para degradação de compostos do petróleo e um conjunto de dados geral para fungos e bactérias baixados (agosto de 2021) do banco de dados acima mencionado, sem inclusão de proteínas contaminantes. Posteriormente, a triagem manual dos resultados obtidos levou em consideração a exclusão de contaminantes comuns encontrados em experimentos proteômicos como por exemplo: queratina, BSA, tripsina e outros.

3.16 CARACTERIZAÇÃO DO PETRÓLEO

A caracterização da amostra de petróleo bruto da bacia do Recôncavo Baiano foi realizada pelo método *whole oil* de acordo com o protocolo adaptado da Environmental Protection Agency 8270 D e 3540 C (EPA, 2007, 1996b). A análise foi realizada em cromatógrafo com detector de ionização de chama (GC-FID) (Agilent®), modelo 7890B, e a cromatógrafo a gás acoplada à espectrometria de massas (GC-MS) (Agilent®), modelo 7890B/5977A, NIST 2011 MS Library (G1033A). O GC-FID apresenta uma coluna capilar de sílica modelo 122-0112 (15 m x 250 µm x 0,25 µm), forno com temperatura inicial de 40 °C e capacidade máxima até 330 °C, taxa de rampa de aquecimento de 10 °C min^{-1} e 12 min de espera. O injetor e detector possuem capacidade para 300 °C, volume automático do amostrador (ALS) de 1 µL, o fluxo da coluna é de

1 mL min^{-1} e o módulo de injeção *split* 20:1 com fluxo de 20 $mL.min^{-1}$ e entrada SS frontal Modo H2 à 3.2876 psi. O CG-MS apresenta uma coluna capilar DB-5ms, Ultra Inert (5 % de fenil/95 % dimetilpolisiloxano, 60 m x 250 µm x 0,25 µm), acoplado com amostrador automático 7693, e detector de massas 5977A. Com fluxo constante 1,2052 $mL.min^{-1}$ com gás de arraste, Hélio. Temperatura da transferline de 280 ºC. O volume de injeção foi de 1 µL, modo de injeção do tipo Splitless a 300 ºC. Uma rampa de temperatura iniciando em 55 °C por 1 minuto, subindo 27 °C por minuto até atingir 300 °C, se mantendo por 6 minutos. As temperaturas de interface e quadrupolo e fonte de íons foram mantidas a 260 °C e 300 °C. A aquisição das amostras foi feita no modo de Monitoramento Seletivo de Íons (SIM). A eficiência da taxa de recuperação dos hidrocarbonetos foi avaliada por meio do padrão p-Trifenil (1000 $ug.L.^{-1}$).

Os dados foram analisados por meio do *software* Agilent MassHunter fornecido pelo fabricante e calibrado com padronização interna de Mix de alcanos C_8 a C_{40} (Sigma-Aldrich®) e p-Terfenil (Accustandard) para hidrocarbonetos policíclicos aromáticos.

3.17 EXTRAÇÃO DE HIDROCARBONETOS EM MATRIZ LÍQUIDA

A extração dos hidrocarbonetos das amostras do experimento solúveis em água ocorreu por meio do metodo líquido/líquido (L/L) baseado no protocolo da United States Environmental Protection Agency, 3510 C (EPA, 1996a). Esse procedimento trata-se da extração do analito na amostra através da homogeneização em solvente de grande afinidade com a fase orgânica (Figura 11). Desse modo, ocorre a adição de 80 mL de amostra (previamente filtrada em algodão) a 60 mL de diclorometano (Merck®) em funil de separação com capacidade para 250 mL. Posteriormente a tampa do funil é posicionada e inicia-se a agitação manual. A homogeneização permanece durante 3 minutos, sendo que são 10 segundos de agitação vigorosa com intervalo de 5 segundos de repouso para eventual liberação da pressão do sistema abrindo a torneira lentamente. Após transcorrido esse tempo, o funil é posicionado na vertical com auxílio de um suporte universal para abertura da tampa e então é mantido em repouso por aproximadamente 10 a 30 minutos até total separação das fases. Uma vez separadas as fases por decantação, é feita a drenagem da fase orgânica para um balão redondo através de um funil contendo algodão limpo e 10 g de sulfato de sódio (Merck®). Dessa forma, o sulfato de sódio vai retirar qualquer remanescente de água e material suspenso garantindo que somente a fase orgânica seja recolhida. Para potencializar a extração o procedimento é repetido por mais 2 vezes.

Ao completar 3 ciclos de agitação e posterior transferência da fase orgânica, as amostras foram concentradas para ± 1,0 mL em rota-evaporador (BÜCHI®), e transferidas para vails de 2,0 mL previamente descontaminados e pesados. Então o material foi aferido a massa e evaporado completamente sob a corrente suave de N_2 e avolumadas com Diclorometano ultrapuro (Merck®) a uma concentração final de 0,02 mg.µL para posterior injeção em CG. Após patronizar a concentração das amostras, aquelas que possuiam volume de solvete inferiror a 60 µL foi preciso usar *inserts* para vials, com capacidade maxíma de 100 µL. O processo de extração e manipulação da amostra ocorreu dentro da capela de exaustão e sem a utilização de material plástico a fim de evitar contaminação por ftalatos. A eficiência de extração foi monitorada pela adição de 10 µL de em solução p-Trifenil (1000 ug.L.$^{-1}$).

Através da análise em CG foram monitorados os alcanos normais do C_8 até C_{40}, os isoprenoides pristano e fitano, os hidrocarbonetos totais (HTP) e as misturas complexas não resolvidas (UCM) e posteriormente os 16 hidrocarbonetos policíclicos aromáticos (HPA) prioritários (Quadro 8). O resultado do teor de HTPs e HPAs foram expressos em mg.L^{-1} e µg.L^{-1}, respectivamente conforme Equação 4 e 5.

Equação 4:

$$HTP_{AMOSTRA}\left(\frac{mg}{L}\right) = \frac{HTP_{vial}\left(\frac{mg}{L}\right)}{Volume_{amostra}\ (L)} \times 0{,}0005$$

Onde:

$HTP_{AMOSTRA}$ = Concentração de HTPs no volume de amostra, em mg.L^{-1}

HTP_{vial} = Concentração de HTPs contida no vial em um volume de 500µL, em mg.L^{-1}

$Volume_{amostra}$ = Volume de alíquota da amostra de água

Equação 5:

$$HPA_{AMOSTRA}\left(\frac{\mu g}{L}\right) = \frac{HPA_{vial}\left(\frac{\mu g}{L}\right)}{Volume_{amostra}\ (L)} \times 0{,}0005$$

Onde:

$HPA_{AMOSTRA}$ = Concentração de HPAs no volume de amostra, em µg.L^{-1}

HPA_{vial} = concentração de HPAs contida no vial em um volume de 500µL, em µg.L^{-1}

$Volume_{amostra}$ = volume de alíquota da amostra de água

Para avaliar o nível de deplecção ou intemperismo do óleo cru dissolvido nas amostras do experimento foram utilizadas as razões tradicionais: Pristano/Fitano (Pr/Ph), Pristano/n-C_{17} (Pr/C_{17}), Fitano/n-C_{18} (Ph/C_{18}) e (HTP/UCM) segundo Steinhauer e Boehm (1992). Além disso foram aplicadas as equações de índices intempérico (ID) fator intempérico (FI) e eficiência de degradação (Oil %), conforme sugerido por Wang e Fingas (1995), Barakat et al. (2001) e Dai et al. (2020), respectivamente e demonstrado nas Equações 6,7 e 8.

Equação 6:

$$ID = \frac{nC_{10} + nC_{12} + nC_{14} + nC_{16}}{nC_{22} + nC_{24} + nC_{28} + nC_{30}}$$

Onde:

C_{10} ao C_{16} = Alcanos linear leves
C_{22} ao C_{30}= Alcanos linear pesados

Equação 7:

$$FI = \frac{nC_{13} + nC_{14}}{nC_{25} + nC_{26}}$$

Onde:

C_{13} e C_{14} = Alcanos linear leves impar e par
C_{25} e C_{26} = Alcanos linear pesados impar e par

Equação 8:

$$Oil\ \% = \frac{C_0 - C_t}{C_0} \times 100$$

Onde:

C_0 = Concentração inicial do petróleo
C_t= Concentração final do petróleo

Quadro 8 – Nomenclatura e respectivos acrônimos dos dezesseis HPAs prioritários da US EPA e suas principais características

Abreviação	Nomenclatura	N° de anéis	Peso molecular ($g.moL^{-1}$)	Genotoxicidade	Carcinogênico
NAP	Naftaleno	2	128,17	Positivo	Possivelmente carcinogênico
ACN	Acenafteno	3	154,21	Questionável	Não avaliado
ACL	Acenaftileno	3	152,2	Questionável	Não avaliado
ANT	Antraceno	3	178,23	Negativo	Não classificado
PHN	Fenantreno	3	178,23	Questionável	Não classificado
FLN	Fluoreno	3	166,22	Negativo	Não classificado
FLT	Fluoranteno	4	202,26	Positivo	Não classificado
BAA	Benzo (a) antraceno	4	228,29	Positivo	Positivo
CHY	Criseno	4	228,29	Positivo	Positivo
PYR	Pireno	4	202,26	Questionável	Não classificado
BAP	Benzo (a) pireno*	5	252,32	Positivo	Positivo
BBF	Benzo (b) fluoranteno*	5	252,32	Positivo	Positivo
BKF	Benzo (k) fluoranteno*	5	252,32	Positivo	Positivo
DBA	Dibenz (a, h) antraceno	6	278,35	Positivo	Positivo
BGP	Benzo (g, h, i) perileno*	6	276,34	Positivo	Não classificado
ICP	Indeno [1,2,3-cd] pireno*	6	276,34	Positivo	Positivo

*Compostos considerados prioritários pela European Council Directive 98/83/EC

Fonte: PubChem, 2021 e USEPA, 2022.

Figura 11 – Extração líquido-líquido de amostras com petróleo. (a) Separação da fase orgânica por decantação. (b) Filtragem do analito e transferência para balão de fundo redondo

A **B**

Fonte: Autora, 2022.

3.18 REMOÇÃO DE CONTAMINANTES E DESCARTE DE RESÍDUOS

Os reagentes utilizados no experimento foram de grau analítico de acordo com o tipo de análise e a água destilada utilizada para a lavagem e preparo das soluções foi proveniente do sistema de purificação (Marconi), modelo MA – 078.

A descontaminação das vidrarias utilizadas no experimento para a eliminação da matéria orgânica ocorreu através do processo de limpeza sob 3 etapas: lavagem em banho de solução Extran (Merck®) a 10 % mantido por 12 horas, lavagem com água destilada por três vezes, e em seguida limpeza com Diclorometano ultra puro (DCM) (Merck®). Quando a vidraria possuía aparência engordurada foram submetidas a etapa adicional antes da solução Extran utilizando a solução de hidróxido de potássio (KOH) a 5 % por meio da incubação em banho de ultrassônico (Elma Transsonic, TI-H10), durante 8 minutos, no modo *Sweep* ou mantidas sob repouso preenchidas pela solução KOH durante 12 horas. Vidrarias menores, tais como pipetas de pasteur e vials passaram também por etapa de calcinação em mufla a 450 °C durante 4 horas, bem como o sulfato de sódio (Merck®). O algodão utilizado na extração de hidrocarbonetos foi anteriormente descontaminado com DCM em aparelho de *Soxhlet.*

A descontaminação microbiológica foi realizada com solução de hipoclorito de sódio a 2 %, em seguida lavadas com água destilada e posteriormente autoclavada a 121 °C a 1 ATM por 15 minutos e submetidas à radiação de luz ultravioleta (UV) durante 15 minutos. O material que não pôde ser esterilizado em autoclave foi submetido à radiação UV durante 15 minutos antes de seu uso. Utilizou-se água destilada e água ultra pura para o preparo das soluções empregadas nesse trabalho. Os ensaios foram realizados em condições aeróbias e os procedimentos realizados em câmara de fluxo laminar previamente esterilizada com álcool a 70 % e luz UV durante 15 minutos.

A vidraria utilizada para análises proteômica foi lavada com solução de Extran (Merck®) a 10 % e o detergente removido com água corrente e posteriormente em água ultrapura. Posteriormente o material foi submetido a banho em solução de ácido nítrico 10 % por pelo menos 1 hora e lavado três vezes com água ultrapura. Em seguida, a vidraria foi lavada com solução 1:1 de água ultrapura (LGC biotecnologia®) e etanol (Qhemis) somente no momento de uso.

Os resíduos biológicos foram autoclavados a 121 °C a 1 ATM por 40 minutos e congelados para transferência em bombonas de polietileno de alta densidade (PEAD), fornecidas

pela empresa responsável. Posteriormente o descarte foi realizado pela empresa RETEC - Tecnologia em Resíduos conforme procedimento padrão de acordo com a legislação ambiental do Conselho Nacional do Meio Ambiente (CONAMA, 2021), atendendo à Resolução nº 358, de 29 de abril de 2005. O material contaminado por óleo, tais como papel e plásticos, foram entregues a empresa AMBSERV - Tratamento de Resíduos, e então incinerados seguindo-se as normas vigentes.

3.19 ANÁLISES ESTATÍSTICAS

Os dados gerados foram tabulados através do Microsoft Excel®, versão 2108 do pacote Microsoft Office 2016® e submetidos a tratamento estatístico por meio do programa *Software R* e *RStudio* (R CORE TEAM, 2021), versão 4.0.5 (2021-03-31) com o pacote Rcmdr (FOX; BOLCHET-VALET, 2020) e posterior produção de gráficos para interpretação e comparação com a literatura atual. Para o desenvolvimento estatístico dos resultados foi utilizada a estatística descritiva para sintetizar os valores em média e mediana, posteriormente foram avaliados segundo a normalidade pelo teste de Shapiro-Wilk e se demonstrarem não paramétricos foram analisados com Teste de Kruskal-Wallis; e quando paramétricos submetidos à análise de variância (ANOVA) para verificação das diferenças estatisticamente significativas adotando o valor de $p < 0,05$. A partir de então, foram gerados gráficos e tabelas.

3.20 APRESENTAÇÃO DOS RESULTADOS

Com o desenvolvimento desta pesquisa foram elaborados 9 artigos para representar os principais resultados a respeito do tema.

1. Mapping the patentary optics of biotechnological processes on bioremediation in Instituto Nacional de Propriedade Industrial with enzyme focus

2. Evolution and overview of hydrocarbon degradation on the enzymatic perspective: a bibliometric review

3. Comparative performance of methods of extracting DNA from filamentous fungi

4. Biotechnological interest microorganisms molecular identification

5. Microbial consortium interaction performance of interest for petroleum degradation

6. Bioprospection of ligninolytic enzymes from marine origin filamentous fungi (publicado anteriormente, APÊNDICE 13)

7. Analysis of functional prediction of the hydrocarbon degradation consortium

8. Oil depletion insight from multiple microbial degradation systems

9. Microbial consortium proteomic response to petroleum hydrocarbons

4 MAPPING THE PATENTARY OPTICS OF BIOTECHNOLOGICAL PROCESSES ON BIOREMEDIATION IN INSTITUTO NACIONAL DE PROPRIEDADE INDUSTRIAL WITH ENZYME FOCUS

4.1 ABSTRACT

Although Brazil has great competence in producing patents for natural molecules originating from plants, microorganisms and/or animals for the bioremediation of contaminated environments, many challenges have yet to be overcome. Thus, this research aimed to map patents deposited in Brazil that hold technologies for the remediation of contaminated environments using microorganisms with an emphasis on enzymes. For this purpose, the database Instituto Nacional de Propriedade Industrial was used, searching for the code B09C1/10 and the European patent database for worldwide comparison. Among the 59 patents filed in Brazil, 41 are of international origins, and of these, 18 were filed by the United States. Deposits were dominated by the industrial sector represented by 56 % and are mainly related to the decontamination of environments contaminated by oil (93 %). Most likely, the lack of the practice of protecting academic research results is an obstacle in the interaction of academia and industry. Brazil has enormous potential for the development of white technology, that is, industrial technology; however, it is still in its embryonic phase and needs greater academic-industrial interaction to reduce obstacles in this area.

Keywords: Biological remediation; Enzyme; Green technology; Patent; Petroleum; White biotechnology.

4.2 INTRODUCTION

The biotechnological process of removing contaminants offers greater safety and less disturbance to the environment, in addition to being an efficient and low-cost tool (DA SILVA et al., 2021). Modification of these processes can generate numerous patents. However, the registration of patents in Brazil, an important indicator of innovation, is a long process (GOUVEIA, 2007).

The term bioremediation can be defined as a biotechnological process in which the metabolism of microorganisms is used for the rapid degradation of pollutants, seeking to reduce their concentration to acceptable levels, transforming them into less toxic compounds (YAKUBU, 2007). Microbiological processes have been reported to be efficient in removing xenobionts (AZUBUIKE et al., 2020), because the organic polluting compounds are used as a source of carbon and/or energy by biodegradable organisms (ABATENH et al., 2017). Different groups of naturally occurring organisms can reduce the contaminants in an efficient and continuous manner that occurs primarily due to the natural adaptation process of the native microbiota of the impacted environment (ANDRADE et al., 2010; VOOLSTRA; ZIEGLER,

2020). In recent years, the bioprocess that uses whole microorganisms has been giving way to the use of microbial enzymes separated from its cells (THATOI et al., 2014). These complex biological macromolecules act as catalysts for various biochemical reactions involved in the catabolic routes of pollutants (KALOGERAKIS, 2017). Although there are limitations in enzymatic bioremediation, such as its structural complexity and low stability, considering that any physical and chemical change results in loss of enzyme activity (NIGAM; SHUKLA, 2015), genetic engineering techniques that can work around some of the restrictions are already available (GUPTA; SINGH, 2017).

Patents within the scope of bioremediation fit the definition of "Green Patents" described by Article 2 of Resolution 283/2012 INPI, with those focusing on environmentally friendly technologies (MENEZES et al., 2016). The patenting of an invention can be limited by institutional obstacles and the small scale of instruments to support innovation (ARAÚJO, 2012). However, these deficiencies in Brazil are prone to reduction, from the moment the Federal Government instituted the production and development of enzymes as a strategic development area (MONTEIRO; DO NASCIMENTO, 2009). Innovative activities are stimulated by two significant determinants, one being quality technological infrastructure and the other the strong protection of public relations (PANDA et al., 2020). Shin and contributors (2016) report that a country with a high level of technology drives exporters to increase exports. It can be said that Brazil is a country that essentially imports enzymes, in addition to presenting a still reduced use of these biomolecules in industrial processes when compared to other emerging countries. Thus, the insertion and consolidation of Brazil as a producer of enzymatic technology is necessary (MONTEIRO; DO NASCIMENTO, 2009).

This work aimed to map patents deposited in Brazil that hold technologies for the remediation of contaminated environments using microorganisms with an emphasis on enzymes to describe the technological maturity of the country.

4.3 MATERIAL AND METHODS

Patent prospecting was carried out under code B09C1/10, from January to February 2019, in the Brazilian database of Instituto Nacional de Propriedade Industrial (INPI) available at: <http://www.inpi.gov. br/>. In addition to patents, they are registered in INPI: industrial designs, brands, computer programs, geographical indications, and topographies to stimulate innovation

and competitiveness for technological and economic development in Brazil. No period of the year was stipulated for the filing of patents at the INPI however, the time range found was used to conduct the search in the foreign database. To incorporate the search, the European patent database (ESPACENET), available in: <https://worldwide.espacenet.com/patent/> respecting the search criteria: Code-B09C1/10; Country Brazil; Year-1996 to 2016. When analysed without country restriction, ESPACENET has more than 8000 patents with this theme, which goes beyond the base's data collection capacity thus, only the total number of patents per country was used for international comparison. The data were collected manually and included information about the patent title, year, country of origin, inventor, the applicant's profile and additional information related to the contaminant and the type of technology used. The identification of the profile of the patent applicant was classified as: University, Company or Individual Candidate. Later, graphs and tables were generated using Microsoft Excel 2016.

4.4 RESULTS AND DISCUSSION

The search for code B09C1/10 returned 41 patents using the INPI database and 52 using the SPACENET database. Of these documents, 24 were duplicate records and 10 did not apply to the study and, thus, were excluded from the analysis. This code refers to the recovery of soil contaminated by microbiological processes or through the use of enzymes, which is equivalent to bioremediation. Table 1 shows the main information extracted from the study in question.

The evolution of the patent filing in Figure 12 shows a total of 59 registrations in the period from 1996 to 2016, with no more recent applications. Then we create a statistical forecast based on the previous data. There was an increase in 2005 and 2009 and record gaps in 2002, 2006 and 2015.

Despite the INPI's general patent application records have been available since 1990, however, for this research, results were only found from 1996 onwards. Probably the patent registration applications were stimulated after the approval of Law n° 9.279/96, which controls the directives and obligations relating to industrial property (CARDOSO et al., 1996). The increase in 2009 may be a reflection of the concession to patent the results of technological research validated in the previous year (CASTILHO, 2001).

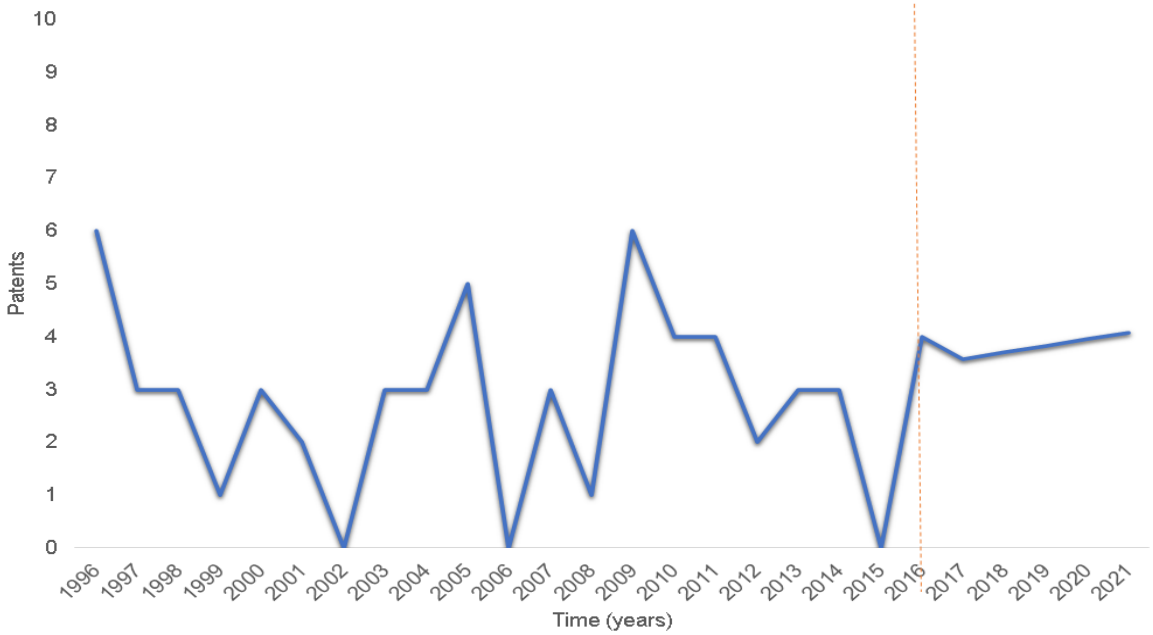
Table 1 - Summary of the parameters investigated in the search for patents filed in Brazil with code B09C1/10 on the INPI and ESPACENET database

Parameters	Occurrence
Patent	59
Language	Portuguese, English, French and German
States	7
Countries	14
Individual candidate	10
Universities	11
Companies	27
Year	1996-2016

Source: Author, 2022.

The record gaps found in 2002, 2006 and 2015 in the following years, it can be considered a common "backlog" in other words, it is conceived as a chronic delay of publication deposits due to bureaucracy and internal operational difficulties of the INPI, in addition to the necessary period of 18 months of secrecy. These pending issues become obstacles to technological innovation, given that they create legal insecurity and discourage investors (GARCEZ-JÚNIOR; MOREIRA, 2017). The Brazil has great competition for the generation of biotechnological products, especially the period called the Brazilian “scientific boom”, where changes were introduced as of 2003 for Política Industrial, Tecnológica e de Comércio Exterior (Pitce) with the Innovation Law in 2004 and the Good Law in 2005 (ARAÚJO, 2012), a fact that can be noticed in Figure 12. However, specialists such as Pacheco (2011), Araújo (2012), Rapini (2013) and Macedo (2017) affirm that the growth of technological innovations, in general, does not appear to be relevant interms of changes, even with the governmental implementation of innovation incentives. A recent report on environmental remediation technologies (BCC 2021a) revealed that the global market reached US$20.290 million in 2019 in the oil and gas industry sector and is immune to the 6.8 % growth of the compound annual growth rate to 2025, which could reach more than US$30 billion. That said, it is estimated that this picture may evolve positively in the following years, according to the linear progression represented after the red line and, mainly, after the environmental catastrophe deliberated by the spill of more than 4 thousand tons of oil distributed in 1000 km of the Brazilian coast in 2019 (GOVERNO DO BRASIL, 2020; IBAMA, 2020).

Figure 12 - Annual evolution of patents deposited in the INPI base with code B09C1/10 and its future perspectives for 2017 to 2021, being that after 2016 corresponds the progression



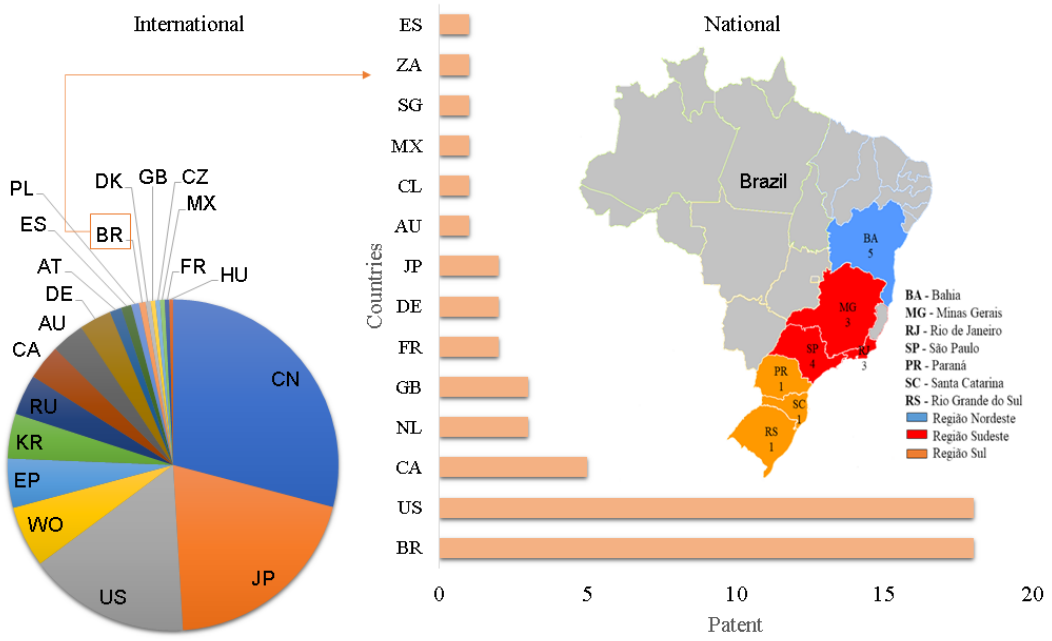
Source: Author, 2022.

The survey of data in the patent databases shows that among the inventing countries the most filed patents are Brazil and the United States's. Brazil stands out with 30.51 %, followed by Canada with 8.47 %, and Holland and the United Kingdom, both with 5.08 % (Figure 13). Generally, deposits made in code B09C1 /10 of foreign origin (69.49 %) were higher than deposits of Brazilian origin (30.51 %). Among the national depositors is the state of Bahia, which appears to be the main contributor. Bahian patents are all linked to biodegradation processes of petroleum hydrocarbons using biosurfactants, protein biopolymers, indigenous consortia or methods of obtaining a biodegradable products (CUNHA et al. 2003; GONCALVES; QUINTELLA, 2013; MOREIRA et al., 2016; QUINTELLA; GONCALVES, 2012). Then there are the states of Rio de Janeiro, São Paulo and Minas Gerais, highlighting the Southeast region. With regard to the international perspective, 8732 patent deposits were found in the ESPACENET database with the same code, and of this amount Brazil is ranks in 14th place. The share of deposits of Brazilian origin represents only 1 %, which is lower than China with 2429 patents (29 %), Japan with 1665 patents (20 %) and the United States with 1318 patents (16 %), countries which also deposit at the INPI base, with the exception of China.

The World Intellectual Property Organization (WIPO, 2020a) points out that the rate of patent applications in the last 10 years is higher in non-residents, a fact that corroborates with the data of this research. The US is among the largest import and export partners products to Brazil (EXPORTGENIUS, 2021), so the congruence of the data suggests that this country may have an interest in the Brazilian market and, therefore, are competitors in this sector. The efforts to forecast trends and changes and the search for technological innovation are methods adopted by companies to remain competitive, staying one step ahead of competitors in the market (DAS VIRGENS et al., 2018). Thus, it is indispensable for competitive intelligence, patent prospecting and data monitoring (PARANHOS; RIBEIRO, 2018). Supposedly, patents deposited in Bahia are linked to the need to manage waste generated from the activities of the oil and biofuel industry, which represents 13.8 % of the industrial sector in the state (CNI, 2017). The geographic predominance of the Southeast region over deposits may be linked to the high concentration of economic activities in the region, as well as the availability of a range of companies that show interest in the development of clean technologies (MENEZES et al., 2016). Due to the maturity of the research centers in this region, the best universities in Brazil are found. It is suggested that Brazil, as an emerging country, is still in the development phase in this field of technology, despite the growing interest on the world stage. China, Japan and the United States have registered patents intensively, conferring the title of the three largest depositors in most technological areas (WIPO, 2020b). Our findings corroborate the research by Rodigues et al. (2020), where the world registration of enzyme patents is dominated by China however, North America has its prominent role with higher revenue from the global enzyme market. Apparently, the topic is of worldwide interest, with the Asian continent dominating the ranking of deposits with values up to 40 times higher than Brazil. The United States, China and Brazil have the same innovation policies aimed at market demand however, the US is a pioneer in adopting these policies, while in Brazil, the initiatives are more recent, because they are based on late industrialization processes (MACEDO, 2017). Thus, it is deduced that, despite having similar policies, these processes are poorly structured in Brazil, generating an effect on the country's technological development.

Notably, when evaluating data referring to patent holders (Figure 14), companies' interest in bioremediation technologies stands out. Companies represent 56 %, followed by universities with 24 %, along with individual candidates with 18 % and research centers with 2 %.

Figure 13 - International and national scenario of the number of patent filings by country of origin in code B09C1/10 in the period from 1996 to 2016.



Source: Author, 2022.

Legend: BR = Brazil, US = United States, CA = Canada, NL = Netherlands, GB = United Kingdom, FR = France, DE = Germany, JP = Japan, AU = Australia, CL = Chile, MX = Mexico, SG = Singapore, ZA = South Africa, ES = Spain, WO = World Intellectual Property Organization, EP = European patent organization, KR = Republic of Korea, RU = Russian Federation, AT = Austria, PL = Poland, CZ = Czech Republic, HU = Hungary, DK = Denmark.

Among the 10 companies represented in Figure 14 with the highest number of patents are DuPont and Zeneca Corp. The participation of Zeneca Corp. is reflected in its three patents referring to soil decontamination by organochlorine products (GRAY; GRAY, 1999; MOSER; GRAY, 1999; GRAY et al., 1999). On the other hand, DuPont presents patents that provide solutions for decontamination of sites by hydrocarbons, through the addition of biological agents or additives. The Universidade Federal da Bahia (UFBA) and Newcastle University (NCL) lead the top of the educational institutions that presenting the highest number of patents in the investigated dataset. The Newcastle University in England exhibited coauthorship, in both patents, with the company Norsk Hydro. It is worth mentioning that public universities dominated the production of patentable knowledge, and only 2 private educational institutions, the University of Pennsylvania in the United States and the Catholic University of Rio de Janeiro in Brazil. The Institut de recherche pour le développement (IRD) was the only representative of a public research institution with one patent. Regarding the individual candidate category, the

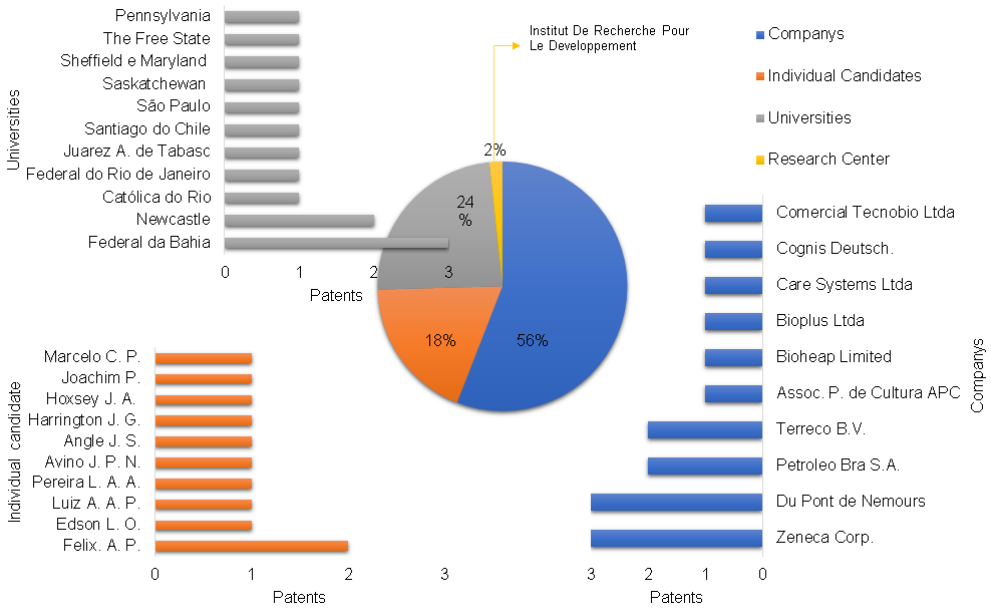
depositor Felix Anthony Perriello stands out with 2 patents on the use of bacteria in the demineralization of petroleum hydrocarbons.

Zeneca Corp, headquartered in Canada, is one of the subsidiaries of Zeneca Ag Produtcs Inc in the agricultural sector, which invests in research to solve problems in agriculture (FORSTALL, 2000). This fact is reflected in their patents, which on this occasion, are related to insecticides that contain dichloro-diphenyl-trichloroethane (DDT), whose widespread are around the world and its high persistence can cause residues in the food chain through the absorption of by the plants (KAFAEI et al., 2020). In Brazil, the use of DDT has been banned since 1985 due to its carcinogenic risk for humans (ANVISA, 2015; 2017) however, its effects may have been observed in subsequent years and thus the interest in patent filing reflected in the 1990s as per the previous documents. On the other hand, DuPont is an American company in the chemical sector recognized worldwide for industrial safety. Although DuPont dominates the world market for the production of industrial enzymes (BCC, 2021b), no patents for environmental use have been found. The preferential use of the bioaugmentation and biostimulation technique can be justified by the fact that the addition of specialized microbial populations, as well as the adjustment of nutrients to the contaminated environment can provide a hydrocarbon removal rate of over 60 %, as seen in the work carried out by Abdulsalam and Olame (2009), they can therefore, be considered excellent candidates for green solutions. Both companies DuPont e Zeneca Corp. check the data of the ranking of depositing countries. These include the United States and Canada which in addition to being the largest depositors, they also represent the companies with the highest number of patents on this topic. With regard to the university, UFBA deposits are linked to researchers with high academic production such as Ícaro Moreira and Odete Gonsalves and who also have other patents in related areas. The coauthorship of the University of Newcastle with Norsk Hydro, a Norwegian aluminium and renewable energy company, allows us to infer that those patents have a business segment. In the meantime, it is believed that teaching and research institutions can play a fundamental role in the generation of new technologies through the application of the scientific base of useful knowledge for the industrial sector (SILVA, 2014). However, due to the lack of a culture of knowledge protection, not all academic research results in patents and, consequently, widens the interaction gap between academia and industry. Thus, the absence of the transfer of technological knowledge to the market becomes an obstacle to society's access do its benefits (BUTTOW; STEINDEL, 2012). Since the results indicated that

public universities dominated the production of patentable knowledge, these data still corroborate the statement that research in Brazil is carried out mainly in public universities (MOURA; CAREGNATO, 2010). Regarding the public research institution, the IRD was the only representative however, it has a worldwide reach. IRD is of French origin but has several international headquarters in West and Central Africa, Asia, Latin America and the Caribbean, among others, where it promotes interdisciplinarity and entrepreneurship with the aim of designing solutions adapted to the challenges that man and the planet face. This includes such changes such as: global, climate change, and loss of biodiversity among others in view of sustainable development. In this way, it justifies its deposit of an innovation patent regarding the decontamination of environments by heavy metals (IRD, 2020). It is also important to highlight that it is expected that the category of individual applicant depositors will be lower than the others mentioned, since the costs for maintaining the patent can be considered relatively high and, therefore, are associated with companies and educational institutions that have greater purchasing power. In this context, the depositor Felix Anthony Perriello filed two patents at the base, however is an inventor who has more than 20 US patents filed with ESPACENET, all linked to the topic of environmental remediation. On the other hand, he only presents 5 scientific publications when identified by the researcher profile ORCID 6507716212, which leads us to believe that he is an inventor of the productive sector affiliated with a company and not an academic researcher (ORCID, 2020). In developed countries such as the US, it is customary to support professionals with a profile of scientific and technological training, as they are considered prominent catalysts for the development of innovative processes (SILVA, 2014). In Brazil, this statement is not contemplated and is probably one of the most relevant criteria that contribute to the fragmentation of economic and technological development in Brazil.

Among the contaminants mentioned in the patents are nickel, cobalt, petroleum and other fuels, fatty oils, residual fats, sanitary and industrial effluents, leachate and insecticide. Such patents were quantified and grouped by type of method of bioremediation and type of contaminant, as shown in Figure 15. Therefore, with regard to the contaminant category is dominated by organic compounds of which approximately 93 % are related to petroleum hydrocarbons. One of the most prominent methods in this research was that of bioaugmentation, followed by bioaugmentation in synergy with biostimulation, and phytoremediation.

Figure 14 - Percentage of the classification categories of the applicant's profile and their respective patent holders filed in the INPI base under code B09C1/10 in the period from 1996 to 2016



Source: Author, 2022.

Regarding the type of mineralizing agent in biotechnological products, patents related to bioaugmentation performed by whole microbiological strains are expressively majority when compared to the application of isolated enzymes. It is known that most of the contaminants are transformed predominantly by biological processes through enzymes and that their use outside the cells has low substrate specificity and can degrade a variety of pollutants (SILVA et al., 2007), however, in this research their direct mention only represents 8.5 %, where half of the documents are from North America.

Among the enzymatic patents is the creation of Alexandre-Junior and collaborators (2016), which deals with obtaining enzymatic complexes of xylanase, lipase, pectinase, beta (ß)-amylase, alpha (a)-amylase, lactase, cellulase and protease obtained through microorganisms of the genera *Aspergillus, Bacillus, Cladosporium, Mortierella, Penicillium* and *Pseudomonas*. This patent was formulated with the aim of treating industrial, agricultural, domestic, urban rainwater and solid waste disposal effluents. Another enzymatic composition found in this research with common characteristics was that of Pereira et al. (2011) where the formulation of a mixture for the production of the enzymes amylase, cellulase, lipase, pectinase and protease and surfactants from fungi of the *Cladosporium, Aspergillus* and *Penicillium* genera was developed. This mixture, in turn, acts as a bioremediation agent for environments impacted by polycyclic aromatic hydrocarbons.

Following the line of degradation of organic compounds are Pereira and Roberto (2010), presented a technique for manipulating plant-derived polymeric enzymes with digestion/biodegradable characteristics, low toxicity, high degree of buoyancy and absorptive capacity, and Laurell and Sicotte (1998) described a composition formed by protein, a bulking agent, and a microbial culture, that is, a combination of bioaugmentation and biostimulation techniques assisted by a biocatalyst.

Addtionally, among the patents is the invention by Gray et al. (2000) which refers to obtaining the haloalkane dehalogenase enzyme that catalyses the removal of fluorine, chlorine, bromine, iodine and acetate atoms for the removal of halogenated contaminants or halogenated impurities from a sample. Thus, this product can be applied to residues that contain in their composition carbon tetrachloride, chloroform, dichloromethane, trichloroethane, dichloroethane, tetraiodocarbon, bromine iodide, bromoform, iodine iodide and other halogenated solvents. Another report was about a method of improving wastewater treatment using *Geobacillus stearothermophilus* to obtain alpha-amylase enzyme. In this sense, the technique is suggested for the treatment of sludge using an enzymatic composition containing alpha-amylase and an additional enzyme that can be esterase, laccase, lipase, protease, hemicellulase, cellulase, oxidoreductase and/or glycosyl hydrolase (DELOZIER; HOLMES, 2005).

Among the mentioned patents, hydrolases, amylase, lipase and protease are commonly used as bioenvironmental solutions for transforming anthropogenic contaminants. We can still observe the predominance of enzymes of microbiological origin with genera of industrial interest, such as *Aspergillus* and *Penicillium*.

Oil represents a great source of energy and wealth sought around the world on the other hand, outside the industrial context it can be considered a threat to human and environmental health (ABTAHI et al., 2020). The complexity of its composition generates major obstacles in the acquisition of an efficient method to meet all the adverse conditions of each contaminated environment and respect national and international environmental regulations (ABTAHI, 2020). These factors may likely give you a greater demand for interest in the production of patents for the remediation of environments and thus justify being a majority among the contaminants found in the search. The purpose of applying microorganisms to the contaminated environment is to accelerate the degradation process and transform a specific toxic compound with the help of the indigenous microbial community to provide adequate conditions for the development of the

degrading microbiota, whereas phytoremediation uses the different mechanisms of certain plants to absorb, volatilize and or degrade the contaminant (WANG et al., 2021). All of these processes occur only due to the action of enzymes present in plants and microorganisms. Currently, there is an awareness that a variety of enzymes are involved in remediation, including laccases and peroxidases, which play a vital roles in this process (KUMAR et al., 2019).

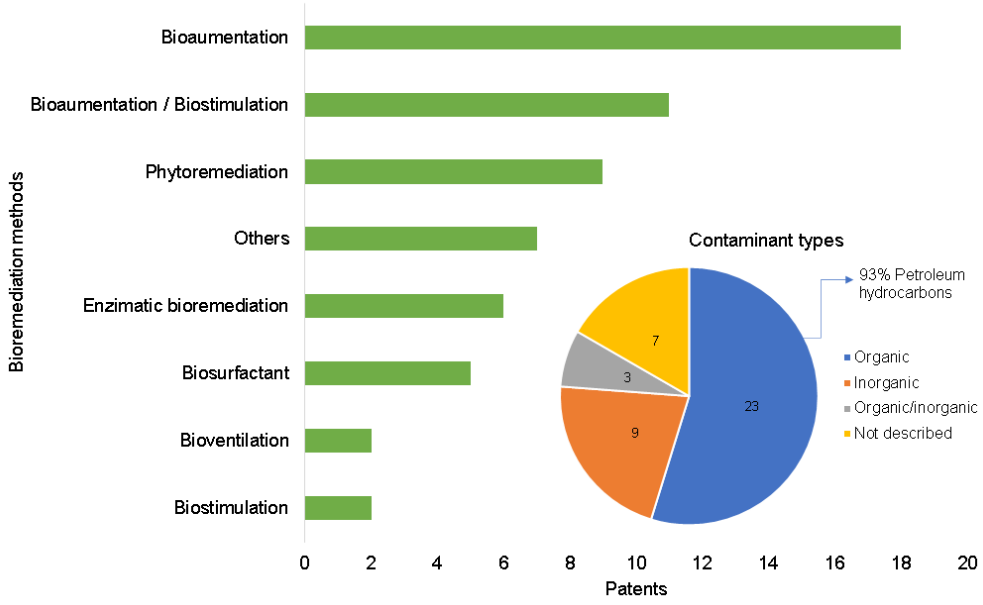
The predominance of enzyme-producing species in this study is congruent with the statement that *Aspergillus* and *Bacillus* are preferentially used to obtain extracellular proteins as they are excellent sources providing approximately 50 $g.L^{-1}$ (PATEL et al., 2017). The use of multienzymes is notorious among the documents found. This is probably due to its high functionality, adaptability and sustainability, which allows the transformation and reduction of the biodegradability time of compounds with expanded application for different types of contaminated substrates and for other sectors of industry (SHI et al., 2018). Apparently, a solution with a single enzyme formulation and whole cell catalysis can establish an important bridge for application in the form of an enzyme cocktail. Thus, synergistic formulations between living organisms are primarily manipulated to obtain suitable materials for the efficient and stable construction of multienzyme catalytic systems (MECSs). On the other hand, patents leave something to be desired relative to techniques associated with the implementation of immobilized enzymes since they are considered the next generation of biocatalysts (CHAPMAN et al., 2018). That said, the discoveries run into a deficit in the patenting of enzymatic technologies.

Regarding the global industrial enzyme market, a recent report published by Business Communication Company (BCC) Research (2021b) reveals a 6.3 % growth trend in the compound annual growth rate (CAGR) for the period 2021 to 2026. This market growth is expected to reach US$6.4 billion in 2021, US$7.0 billion in 2023 and US$8.7 billion in 2026. Thus, market projections for enzymes can be considered one of the development indicators of biotechnological processes with growing worldwide interest. In Brazil, the demand for consumption of enzymes is the majority with regard to Latin American countries, which configures the need to have enzymatic technologies as a necessary reality and not just a promising alternative however, the import rate is still higher than exportation (MONTEIRO; SILVA, 2009). That said, enzymatic technology in the country is significantly behind schedule, even though the region is home to great biodiversity that provides an abundance of renewable raw materials for the production of biocatalysts and viable technologies for the generation of

bioproducts on a large scale (POLITZER et al., 2006). To reverse this situation, it will be necessary for the country to invest in technologies aimed at overcoming the challenges associated with the implementation of enzymatic catalysis.

In this context, it can be said that studies on enzymatic remediation are still under development, and the mechanisms of action and bioprospecting to identify producing microorganisms are not fully understood. However, this remediation strategy has enormous potential and importance for greater efficiency in bioremediation. According to Monteiro e Silva (2009), white technology is a very promising area with regard to environmental control. Currently, the global trend is towards environmental responsibility, where the effect of producing less pollution and generating more decontamination processes is to replace chemical processes with enzymatic processes.

Figure 15 - Bioremediation method and number of contaminant classifications of patents filed in the INPI base under code B09C1 / 10 from 1996 to 2016



Source: Author, 2022.

4.5 CONCLUSION

Most patents on the INPI basis are generally originate from foreign countries, mainly from the United States. It is evident that the contamination of environments by petroleum hydrocarbons is a global concern given the predominance of patenting methods aimed at transforming these compounds. Processes with the use of microorganisms are a trend among the available methods, however the direct use of enzymes is scarce. The gap in Brazil in relation to

enzymatic technology for applications in bioremediation processes was proven in this research, despite efforts from the Southeast region.

Patents from industrial environments are mostly for the area of recovery of contaminated environments. However, the technology transfer mechanism based on university-enterprise interaction has not been observed from the data collected in this study. Many studies may be confined to the laboratory and are not tested for production in a semi-industrial environment due to a lack of incentives. The support of companies would certainly strengthen progress in this sector.

Finally, in tune with the needs of the world market for enzymes, the investigation of multienzyme catalytic systems and the stabilization of enzymes by encapsulation is an opportunity. The production and patenting of these bioproducts would certainly increase the Brazilian commercial and economic representation in the international scenario, also bringing social and environmental benefits. For this, it is necessary to improve the training of professionals capable of generating technological innovation and recognizing the mechanisms for protecting the knowledge generated, as well as investing financially so that Brazil can advance in the development of white technology.

5 OVERVIEW OF THE EVOLUTION OF HYDROCARBON DEGRADATION FROM THE ENZYMATIC PERSPECTIVE: A BIBLIOMETRIC REVIEW

5.1 ABSTRACT

In the last decade, the dioxygenase enzyme has been cited in the literature as an important tool in the hydrocarbon degradation process. In this context, we investigated trends and discussions regarding the degradation of hydrocarbons by the dioxygenase enzyme and present an overview of the dynamics of research development in this area to guide the scientific community. A bibliometric analysis from 1990 to the first half of 2020 was implemented, focusing on articles and patents from the Scopus database. The results showed that 526 articles were published in 164 journals. Among the journals with the highest number of publications were Applied and Environmental Microbiology and Journal of Bacteriology, both with an impact factor above 4. The articles were published by a group of 1855 authors, the most prominent of which were Zyltra GJ and Cerniglia CE. The patents on the subject totaled 3160, covering various industrial applications. We found that studies related to the degradation in sediment and water to subsidize the application of bioremediation processes were lacking; this area has grown in recent years and the development of patents is encouraged.

Keywords: bibliometric prospecting, biodegradation, dioxygenase, enzyme, petroleum, PAH.

5.2 INTRODUCTION

The accumulation of persistent organic pollutants is one of the main environmental contamination problems worldwide. Industrial activities and technological advances in most production sectors contribute to the increasing release of polluting compounds that are highly toxic, bioaccumulative and resistant to physical, chemical, photolytic and biological degradation (BONALUMI, 2010).

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants primarily generated during the incomplete combustion of organic materials (e.g., coal, oil, and wood). Emissions from anthropogenic activities predominate; however, some PAHs in the environment originate from natural sources, such as open fires, natural losses or infiltration of petroleum or coal deposits, and volcanic activity. The main anthropogenic sources of PAHs are as follows: residential heating; coal gasification and liquefaction; carbon black, pitch and asphalt production; coke and aluminum production; catalytic cracking towers and related activities in oil refineries; and motor vehicle exhaust (ABDEL-SHAFY et al., 2016).

Bioremediation is a biotechnological strategy that leverages remediation techniques for contaminated sites. It is based on the use of microorganisms, or their enzymes, to reduce or

eliminate environmental hazards contaminating inert substances (BONALUMI, 2010). In this process, biomass is generated that can be incorporated into humus or converted into CO_2 and H_2O, representing the elimination of hydrocarbons and the generation of products that are inert from a toxicological point of view (SILVA, 2008). The specific properties and functions of enzymes determine whether they can be applied in different biotechnological contexts: environmental bioremediation, biosynthetic processes or food production (PIUBELI, 2011).

The use of enzymes is gaining increasing commercial value because enzymatic processes are a clean technology, and their application saves water, energy, and chemicals and reduces the cost of effluent treatment when compared to conventional chemical methods in industrial processes (SANTOS, 2016). The dioxygenase enzyme, for example, is related to the degradation of alkanes and aromatic compounds, a process that occurs through the incorporation of oxygen atoms (O_2) into the aromatic ring; thus, the chemical reaction is destabilized, and the opening of hydroxylated aromatic rings occurs (PIUBELI, 2011; SILVA, 2008). Various filamentous fungi and bacteria can yield satisfactory results applying this process to the detoxification of oil and its derivatives (VIEIRA, 2016).

Bibliometric review is an analysis that is important for understanding the dynamics of the knowledge of a specific topic. Based on this understanding, it is possible to identify national and international collaboration networks, map the evolution of new fields in science and technology, and determine the internal logic of science development (RUAS; LIMA; PEREIRA; 2014). In this work, we analyze trends and discussions related to the degradation of hydrocarbons via the dioxygenase enzyme through a bibliometric review of published scientific articles to guide the scientific community regarding the dynamics of research on this topic to provide useful information for further studies. A summary of the scientific knowledge on this topic and an overview of publications is provided.

5.3 MATERIAL AND METHODS

The literature review was carried out using a bibliometric approach. To search for scientific sources from 1990 to 2020, the Scopus database (https://www.scopus.com) was used. There are several databases available for performing a review of studies, such as Google Scholar, SciELO, GeoRef, Scopus, SpringerLink, Science Direct, Web of Science (WOS), and Wiley; however, Scopus and WOS stand out as the largest databases in the world in terms of content

volume (CHADEGANI, 2013). The Scopus and WOS databases differ in terms of scope, coverage policies and data volume, but they are similar with regard to the number of citations from countries and their respective classifications, since country outcomes and impacts are correlated (ARCHAMBAULT et al., 2009). Research by Chadegani et al. (2013) and Mongeon and Paul-Hus (2015) compared the Scopus and WoS databases. They found that WoS had strong coverage dating back to 1990 and most journals included were written in English. Scopus covered a greater number of journals and offered access to over 22,000 journal titles and over 27 million patents (ELSEVIER, 2017). Based on these findings, the Scopus Preview database was selected for performing the bibliometric analysis.

The following terms in English were defined as descriptors in the virtual library: dioxygenase, hydrocarbon, and degradation. To guarantee the specificity of the query, a search equation was used to search titles, abstracts, and keywords for the following Boolean terms: dioxygenase AND hydrocarbon AND degradation. A filter was used to restrict the search to full and review articles and a criterion was specified to exclude journals in the fields of medicine, nursing, art, economics and physics.

Data on the number of articles, journals, keywords, authors, average citations per articles and countries were exported in CSV format. Sublime Text software version 3.2.2 was applied to remove duplicates and the data were also selected as recommended by the main items for reporting systematic reviews and meta-analyses (PRISMA) (GALVÃO, PANSANI, HARRAD, 2015). Subsequently, the data were processed using VOSvier Software version 1.6.6 (www.vosviewer.com) and R software, RStudio, version R 4.0.2 (R CORE TEAM, 2020), with the bibliometrix package (ARIA; CUCCURULLO, 2017) and the Biblioshiny tool. The keyword network was constructed with the minimum number of occurrences set to 5, and the minimum number of manuscripts for collaborating countries set to 5. To complement the data, patents during the same time period were searched using the same set of keywords on the “Patents” tab in the Scopus database and the data were collected manually.

5.4 RESULTS AND DISCUSSION

Table 2 presents the descriptive analysis of the main bibliometric parameters investigated on hydrocarbon degradation by dioxygenase. The 526 articles were published in 164 journals from around the world. The articles were published by a group of 1855 authors, representing an

average of 3.52 authors per article. Among those authors, 50 countries were represented, meaning the topic was investigated in approximately 25.9 % of the total number of countries in the world.

Table 2 - Main information on findings on hydrocarbon degradation from an enzymatic perspective from 1990 to 2020

Elements	Quantity
Articles	526
Journal	164
Keywords	1055
Authors	1855
Countries	50
Patents	3160

Source: Author, 2022.

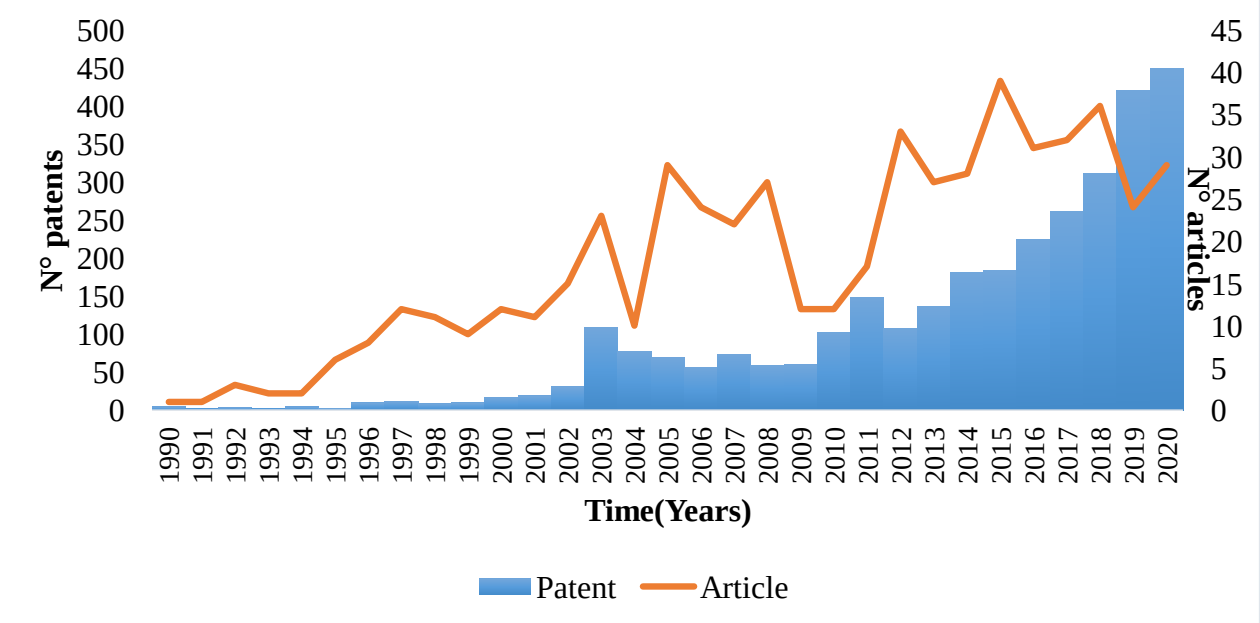
Figure 16 shows the articles published over 30 years, with an average of 17.5 articles per year. The number of publications has increased over the years. Due to the increasing curvature relative to the number of empirical and theoretical studies on hydrocarbon degradation by the enzyme dioxygenase, the continuation of research in this area is warranted. The first studies were reported in 1970 along with the creation of the two-dimensional electrophoresis technique (O'FARREL, 1975); then, they became more numerous in 1990. Since that time, there has been exponential growth in the number of studies, likely due to the emergence of the first genome sequencing technologies and investigation of the human genome project (DE CASTRO REINACH, 1990). The highest peaks in publication represented in the graph can be explained by the emergence of new research technologies, such as the use of mass spectrometry (MS) at the beginning of the 20th century (HAGER, 2004).

During this period, the most cited articles were: Cerniglia (1992) with 1412 global citations; Harayama, Kok and Neidle (1992) with 380 citations; Meckenstock et al. (2004) with 333 citations; Habe and Omori (2003) with 282 citations; and Siciliano et al. (2001) with 273 citations. Only the last one mentioned (SICILIANO et al., 2001) is a research article related to endophytic bacteria containing catabolic genes for hydrocarbons and their dependence relationship with the plant and the contaminant. Cerniglia's article describes the biochemical principles underlying the degradation of polycyclic aromatic hydrocarbons and their catabolic pathways, as well as the main bacteria and metabolites involved in the process. The work by Harayama, Kok and Neidle is focused on the degradation of pollutants from oxygenase enzymes and the specific actions of each system. The stable isotope analysis approach to understand the functioning of aerobic and anaerobic biodegradation of hydrocarbons and chlorinated solvents

and the main related technological advances are explored in the research by Meckenstock and colleagues. Habe and Omori discuss genes involved in the degradation of polycyclic aromatic hydrocarbons, including the structure-function and evolutionary relationships of enzymes. These are classic works that mark the beginning of the era of the omic sciences in the investigation of hydrocarbon biodegradation mechanisms using different approaches. Thus, these articles form a theoretical foundation rich with valuable information that leads us to believe that there is interest on the part of publications and researchers in deepening the research questions in this area.

Patents are encouraged both in industrial and academic areas. That is why it is important to analyze their appearance over the years in order to gauge future expectations for the market. In Figure 16, an upward trend in the volume of patent publications is evident, which may be an incentive to continue developing patents in the area. Of the patents found, more than 80 % are from the United States Patent and Trademark Office (USPTO), which took the lead in patent filings. The patents in this area are centralized in the interests of the American industry, with a concentration of high technology companies. This confirms the ranking of the most innovative countries in the world in 2019 and 2020 in which the US was ranked in the Top 10 (BLOOMBERG, 2020).

Figure 16 - Evolution of publications of articles and patents from 1990 to 2020 on hydrocarbon degradation from the enzymatic perspective



Source: Author, 2022.

It is essential to identify the main journals within the scope of the research to highlight the best sources of valuable information for the topic in question. As shown in Table 3, Applied and Environmental Microbiology and Applied Microbiology and Biotechnology are among the ten journals with the greatest number of publications. Both have been included in the top 25 % of journals covering the same field and are, therefore, classified in the Q1 category of the international classification of the JSR (2021), in addition to having a good impact factor. Applied Microbiology and Biotechnology is second in terms of number of publications, the Journal of Bacteriology has the greatest number of citations based on only 14 published articles. The greatest relative citation index is given to the Journal of Bacteriology, with an average of 147.07 citations per article. The citation index is more crucial than the total number of publications since a greater number of citations indicates that a journal provides more relevant studies. Furthermore, if the objective in publishing research is to reach a greater number of readers, there is evidence that the most cited journals likely attract many researchers and, consequently, have a greater chance of dissemination. Thus, based on the criteria described in Table 3 and the information discussed here, Applied and Environmental Microbiology and the Journal of Bacteriology are good candidates for future investigations and article submissions on the topics of bioremediation, biotechnology, microbiology, industrial microbiology and geomicrobiology. However, the other journals should not be overlooked.

Table 3 - Main journals that published on hydrocarbon degradation from the enzymatic perspective during the period 1990 to 2020

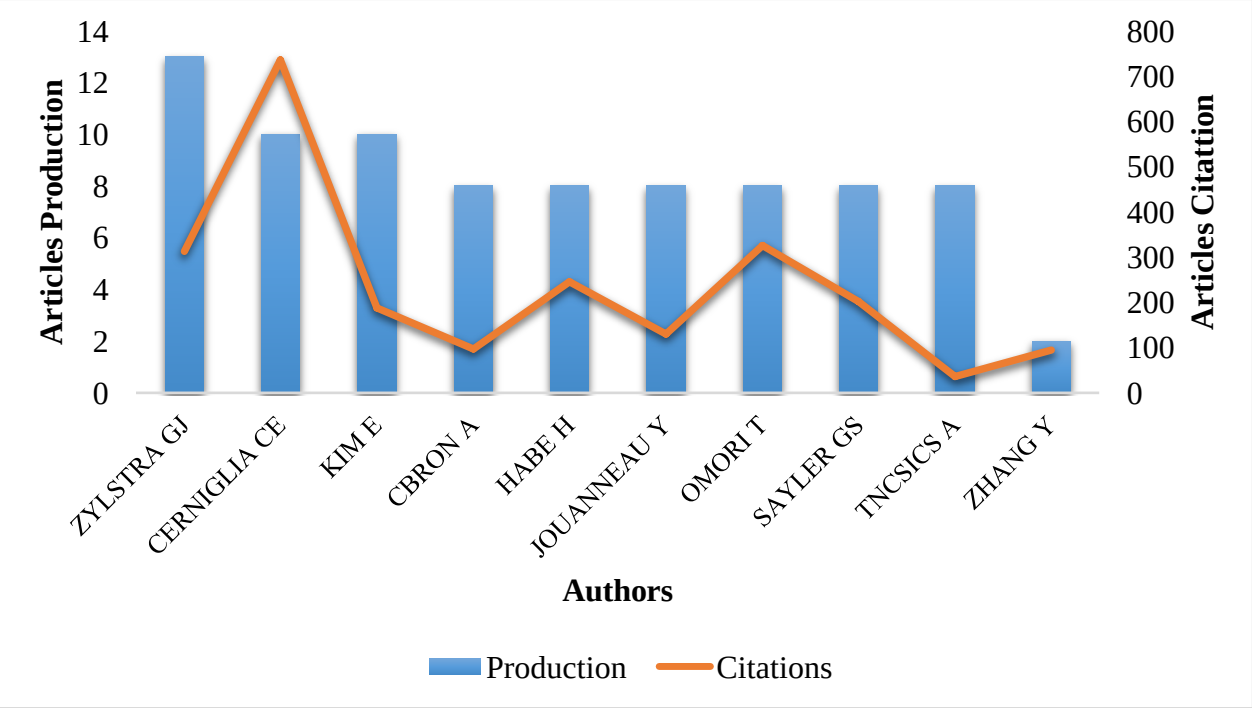
Journals	Quantity	Citation	Relative Citation	h-Index* (2020)	Impact factor **(2020)
Applied and Environmental Microbiology	45	3000	66,66	324	4.792
Applied Microbiology and Biotechnology	29	761	26,24	221	4.813
Journal of Hazardous Materials	20	297	14,85	284	10.588
Environmental Science and Technology	18	561	31,16	58	7.653
Biodegradation	17	477	28,05	77	3.909
International Biodeterioration and Biodegradation	17	289	17,00	103	4.320
Journal of Bacteriology	14	2059	147,07	246	3.490
Chemosphere	14	415	29,64	248	7.084
Fems Microbiology Ecology	11	346	31,45	155	4.194
PloS ONE	11	73	6,63	332	3.240

* Data taken from the Scimago Journal & Country Rank (JSR) portal; ** Data taken from the Journal Citation Reports (JCR) portal.
Source: Author, 2022

In Figure 17 the ten researchers who published the most within the scope selected for this bibliometric review are listed. Gerben J. Zylstra (GJZ), PhD, is at the top of the list with 13 published articles, followed by Carl E. Cerniglia (CEC) and Eungbin Kim (EK), both with 10 articles. Following them are Aurélie Cébron, Hiroshi Habe and Yves Jouanneau, all with 8 articles. GJZ authored the most publications in this theme/period. The publications were mainly in the journal Applied and Environmental Microbiology and many were coauthored by Kim, E., including research that used a combination of whole-cell protein analysis of *Rhodococcus* sp. for the identification of dioxygenases involved in the metabolism of oxylene, toluene and ethylbenzene (KIM et al., 2004). The works of these authors are focused on detailed biochemical, physiological and molecular genetic investigation and on the catabolic pathways of degradation of polycyclic aromatic hydrocarbons by the microorganisms *Burkholderia cepacia*, *Comamonas testosteroni*, *Beijerinckia* sp. and mainly in applications with *Rhodococcus* sp. and *Sphingomonas yanoikuyae*. On the other hand, Carl Cerniglia, PhD is a senior scientist whose work was cited the most and his research focuses on the use of molecular tools for the characterization of hydrocarbon degradation, such as phthalate, phenanthrene and anthracene in *Mycobacterium* sp. In his work, he points out the presence of nidA and nidB genes that encode the alpha and beta subunits of the aromatic ring hydroxylating dioxygenase in *Mycobacterium* species capable of degrading polycyclic aromatic hydrocarbons and therefore can be used as a source of these genes (BREZNA; KHAN; CERNIGLIA, 2003). This author has more than 300 publications, raising his H-index to 69 (WOS, 2021). According to Author Impact Beamplot (SZOMSZOR, 2021), the normalized citation percentile range and mean citation of his work are 0th-100th and 65th, respectively. Based on these metrics, both have an average citation above 60 and are recommended reading for knowledge of the topic discussed; however, they are not the most current.

The countries that stood out the most are shown in Figure 18. The bibliometric analysis showed that the United States, China and Japan have prominent positions, with 45.2 %, 42.9 % and 20.5 % of publications in co-occurrence and 19 %, 17 % and 8 % in relation to the corresponding author, respectively. The countries that contributed the most to the literary production on this topic are also the largest oil producers; the United States is first in the ranking, with a production of 19.5 million b/d, representing 19 % of world production, and China is in fifth, with production of 4.89 million b/d, contributing 5 % of world production (CBIE, 2020).

Figure 17 – Main authors who published the most on hydrocarbon degradation from the enzymatic perspective in the period from 1990 to 2020



Source: Author, 2022.

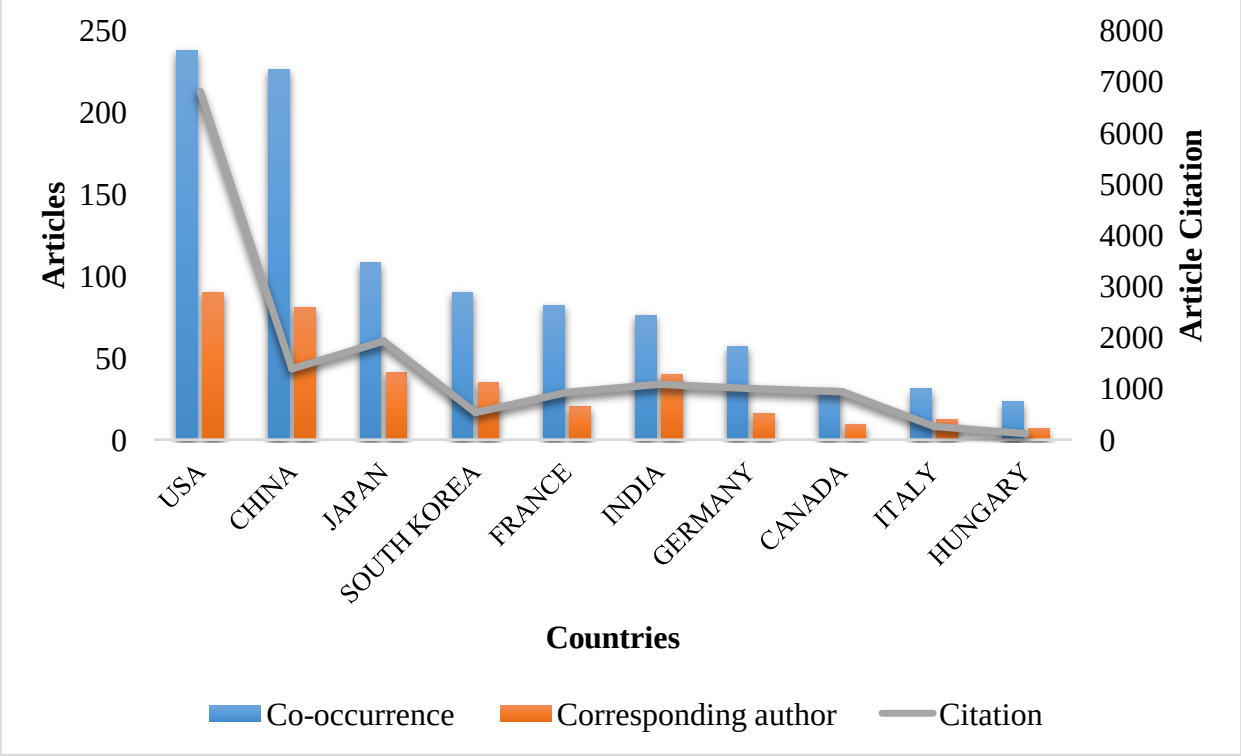
In addition, these countries are listed among the 20 locations with the largest number of oil spill accidents in the International Tanker Owners Pollution Federation Limited (ITOF) (ITOPF, 2018), with accidents such as that of the Exxon Valdez in the US and SANCHI in China. Although the global trend in oil spills by tankers has decreased considerably from 1970 to 2020, oil trade has grown exponentially over that same period (ITOF, 2020). The accidental contamination of environments due to leaks from oil storage tanks, disposal of coke plants and gas plants, and the ineffective decommissioning of facilities (GHOSH; MUKHERJI, 2020) are intrinsic to the growth of these activities. In view of this, the US interest in this area is due to the need for economic activity in the country centered on the production of fossil fuels. In addition to the above facts, it is also considered a developed country and, therefore, has stable knowledge of remediation technologies in areas impacted by hydrocarbons.

In China, the beginning of the 21st century was marked by the accelerated development of the recovery of coastal lands affected by contaminants (WANG et al., 2020). The Pearl River Delta in southern China, which spans the territories of Hong Kong and Macao, is an environment heavily damaged by persistent organic pollutants, including organochlorine pesticides, butyltin,

polychlorinated biphenyls, dibenzofurans and hydrocarbons (FU et al., 2003), which have become the target of studies. The work of Zhou et al. (2020) revealed that seven isolates from Pearl River estuary sediment belonging to the *Roseobacter* clade have versatile efficiency in degrading pyrene, phenanthrene and benzo[a]pyrene through a cometabolism pathway.

In Japan, there are more than 900,000 potentially contaminated regions historically exposed to polluting compounds; among them, 50 % are contaminated with heavy metals and volatile organic compounds (VOCs), including hydrocarbons (ZHANG; YOSHIKAWA, 2020), creating high demand for bioremediation techniques. Even more than 20 years after the heavy oil spill off the coast of Japan by the Nakhodka tanker, the damage can still be observed; Tazaki et al. (2018) found crystallized organic compounds and microbiota degrading hydrocarbons. Detailed investigations of the microbial weathering processes of petroleum in this region support natural purification methods, especially with regard to the bioaugmentation technique using indigenous bacteria due to adaptability to the pollutant's toxicity.

Figure 18 - Top countries that published the most on hydrocarbon degradation from the enzymatic perspective in the period from 1990 to 2020



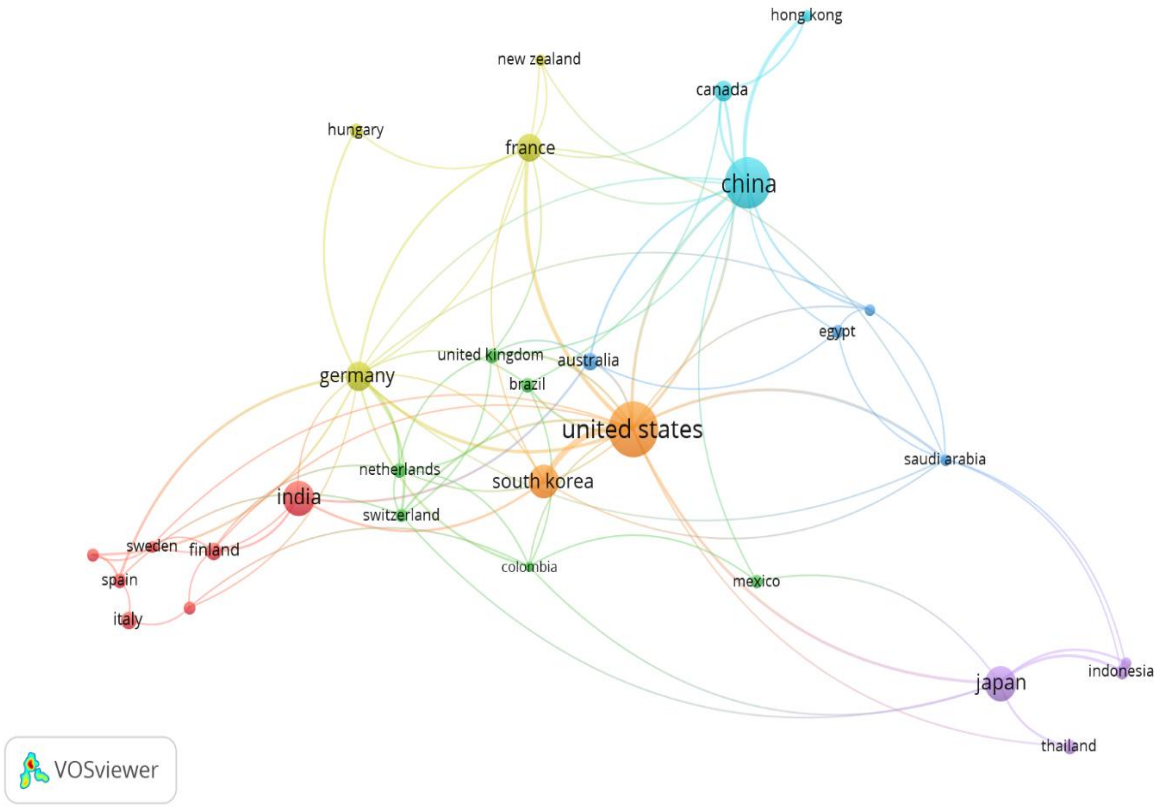
Source: Author, 2022.

Figure 19 shows the 6 main groups of connected countries. The group colored blue includes China with the greatest degree of connection, representing more than 20 articles produced in collaboration with Canada, Egypt, Australia, Saudi Arabia and others. The United States and South Korea are part of the orange group, with more than 9 publications produced by international coauthorship. In the red group, India has approximately 6 documents with connections to several European countries, such as Finland, Spain, Italy and Sweden. Represented in lilac, Japan has just over 4 articles with connections to countries on the same continent, Thailand and Indonesia. The green group includes the countries with the fewest publications; Brazil has few published articles on the subject, which indicates the need for research evolution in the area. However, it has a connection with the United States, Germany, Canada, the Netherlands and Colombia, which may favor the emergence of new partnerships for future publications. The connections between countries may be related not only to the experience in the field of the two countries, but also to the strength of academic-trade agreements. Partnering with the country that has the greatest demand for or expertise on the topic is beneficial for carrying out scientific exchange; the United States is a good partner because in addition to having the largest number of publications, its works are also the most frequently mentioned, with 6803 citations. In addition, since the English language is considered the most widely spoken in the world in relation to the number of speakers (GHOSH, 2020), selecting countries where English is an official language can facilitate communication, as well as the exchange of information. In light of this, partnerships formed through researcher exchange could be the beginning of an advance in publications.

The analysis of the most searched keywords provides a simple summary of what has been studied over the years. Despite the recommendation of Zhang et al. (2016) to use keywords Plus for bibliometric reviews, the author's keywords were used to focus on more specific terms related to the topic. As seen in Figure 20, the term biodegradation is at the center of the study connections and appears 123 times in co-occurrence with the terms bioremediation and dioxygenase, which are mentioned 45 and 42 times, respectively. In later years, it appears with the additional term metabolites, which indicates that “omics” approaches began gaining strength in this area of research. In the last three decades, disadvantages of traditional approaches to the study of microbiology have been pointed out; therefore, the omics sciences have become a

powerful and creative tool for different fields of application, including studies on the bioremediation of polluted environments (PÉREZ-LIANO et al., 2018).

Figure 19 - Network of collaborating countries and their connections on hydrocarbon degradation from the enzymatic perspective in the period from 1990 to 2020



Source: Author, 2022.

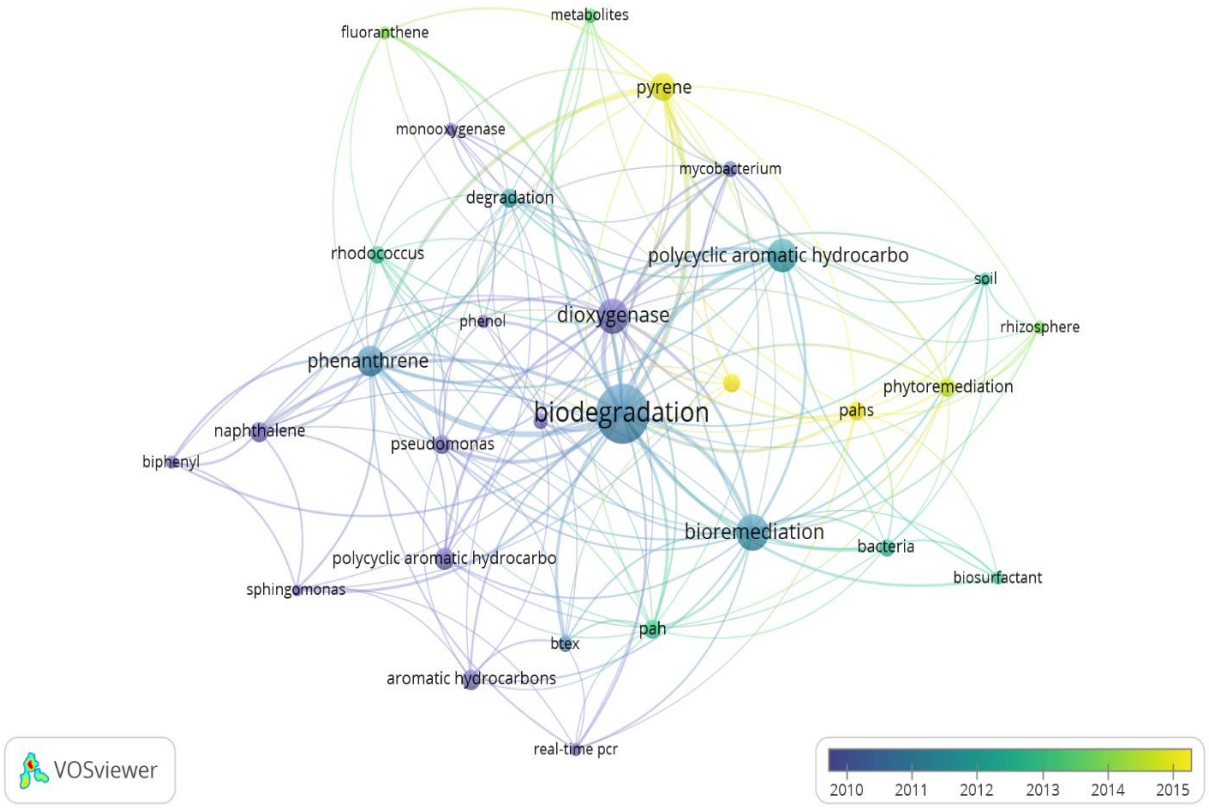
In this sense, the term metabolites can be linked to metabolomic studies, which investigate the primary and secondary metabolites produced by microorganisms under specific conditions and consequently provide useful information regarding the metabolic fluxes and pathways involved in the biodegradation of xenobiotic compounds (SHARMA; SHUKLA, 2020). The 10 documents found with this keyword investigate residual metabolites from the degradation of polycyclic aromatic hydrocarbons (PAHs) by bacteria or fungi or by mixed microbial consortia. In these studies, metabolomics was not the central approach of the study; it was used as a confirmatory method to support the potential of the microorganism tested in the PAH degradation process. Thus, the fundamental role of metabolomics in bioremediation studies is evident.

Among the most current keywords represented in yellow in Figure 20 are polycyclic aromatic hydrocarbons, PAHs, pyrene and phytoremediation. Polycyclic aromatic hydrocarbons or PAHs are considered persistent organic pollutants (POPs) that have a combination of two or more benzene rings in their structure (LUO et al., 2020). Various physical and chemical methods have been studied to solve the problems caused by PAH contamination (KASHYAP et al., 2020). However, there is evidence that physical methods transfer the pollutant elsewhere, rather than degrading it and chemical methods can generate other pollutant compounds as byproducts of degradation (KASHYAP et al., 2020). Methods that are more economical and less invasive have gained great visibility, including phytoremediation where plants are used to clean contaminated environments (HUANG et al., 2019). The synergy between plants and microorganisms enhances the degradation of organic compounds, as seen in the work by Iqbal et al. (2019); in that study, more than 45 % of total hydrocarbons were removed from oil. It is likely for this reason that the term phytoremediation is mentioned among the keywords. The term phytoremediation is also associated with the terms soil, rhizosphere, bacteria, biosurfactant and PAH. However, the terms sediment and water matrices are not mentioned, indicating a possible focus of the studies. Among the 7 documents found with the keyword biosurfactant, the works by Muthukamalam et al. (2017) stand out; the bacteria *Corynebacterium aurimucosum*, *Acinetobacter baumannii* and *Microbacterium* sp. removed hydrocarbonoxydans from contaminated soil over 13 days with emulsification rates of 57 %, 45 % and 69 %, resulting in TPH degradation rates of 74 %, 52 %, and 43 %, respectively. In the study by Khemili-Talbi et al. (2015), the halophilic bacterium *Natrialba* sp. isolated from saline water exhibited emulsification rates of 86.3, 83.3 and 81.8 % in the absence of cells and 82.5, 78.2 and 79.1 % in the presence of cells in phenol, naphthalene and pyrene, respectively. In Kumari et al. (2012), *Pseudomonas* sp. BP10 acquired from crude oil and *Rhodococcus* sp. NJ2 isolated from contaminated soil exhibited emulsification indices of 75 % and 35 %, respectively, contributing 43.6 % and 32.4 % to the degradation of TPH at 30 days. Generally, the biosurfactants studied are of microbial origin; however, in the study by Blyth et al. (2015), it was possible to produce biosurfactants derived from red ash trees, which resulted in removal of 16.2 % of TPH and 6 7 % of PAH in 30 days.

The words *Pseudomonas*, *Rhodococcus*, *Mycobacterium* and *Shpingomonas* are highlighted, indicating that they are a genus of microorganisms that produce oxidoreductase enzymes and have great potential for the mineralization of hydrocarbons. In addition to

dioxygenase, the enzyme monooxygenase is also mentioned; both catalyze oxidation–reduction reactions, but monooxygenase acts on n-alkanes through the addition of a hydroxyl to the terminal methyl group (CHRISTIAN et al., 2020), whereas dioxygenase catalyzes the incorporation of one or two oxygen atoms in organic substrates (IACOPINO et al., 2020). The most cited pollutants are: fluoranthene and phenol, which are associated with *Pseudomonas*; phenanthrene, naphthalene, and biphenyl, which are associated with *Sphingomonas*; and pyrene, which is associated with *Mycobacterium*. PAHs and BTEX did not appear with any genus of degrading microorganisms. These are likely the compounds with inherent hydrocarbons commonly found in anthropogenic activities such as the pharmaceutical industry, petroleum activities, paint industry, plastic and pesticides, among others.

Figura 20 - Author's keyword network and their connections according to year of publication on hydrocarbon degradation from the enzymatic perspective in the period from 1990 to 2020



Source: Author, 2022.

5.5 CONCLUSION

The bibliometric review of the literature on hydrocarbon degradation from an enzymatic perspective showed that there is a well-founded basis for study. In this sense, some authors and journals stand out; the researchers Cerniglia, C. E and Zylstra, G. J, and the journal Applied and Environmental Microbiology may contribute to advancing future research.

Based on the analysis of keywords, there is evidence that this field of knowledge has gaps, such as research related to new targets including the mangrove sediment matrix and air, as well as the investigation of enzymes from fungi and cyanobacteria. The United States is considered the center of research and, therefore, the source of the main studies; connections with the United States would be favorable for collaboration.

The importance of the subject is highlighted by an exponential increase in the number of studies and the number of patents focused on the subject. Finally, the topic remains relevant today with several important applications and it is a field with potential for further study.

6 COMPARATIVE PERFORMANCE OF METHODS FOR EXTRACTING DNA FROM FILAMENTOUS FUNGI

11.2 ABSTRACT

The method of DNA extraction is crucial for obtaining quality material for molecular identification. Therefore, we seek an procedure that is capable of attending to the particularities of the sample. The present work aims to compare five DNA extraction procedures applied to filamentous fungal samples in relation to the quality and quantity of material extracted. The protocols tested were as follows: the Norgen® isolation kit, Maxwell® isolation kit, Norgen® kit modified, protocol using Brazol® and guanidine thiocyanate, and manual protocol. The concentration and purity of DNA were evaluated by fluorescence and spectrophotometry. The quality of the samples was evaluated by 2 % agarose gel electrophoresis and was tested with the amplification of the ITS gene by PCR. From the results obtained, the Norgen® isolation kit is suggested as the most appropriate, since it obtained DNA of good quality and regular quantity.

Keywords: DNA extraction, extraction protocol, filamentous fungi, fungus DNA,

11.3 INTRODUCTION

The extraction of fungal DNA can become a complex task due to the nature of the sample and not all protocols are sensitive enough to obtain satisfactory results (GRIFFIN et al., 2002). Molecular identification is closely linked to the extraction efficiency and DNA quality. Problems at this stage create subsequent problems, so it is essential to investigate adequate protocol for each situation (SILVA-JUNIOR, 2001).

The rupture of the plasma membrane can be laborious because certain microorganisms are resistant to enzymatic digestion and chemical degradation (MAZZIOTTI et al., 2017). Filamentous fungi have elements in their cell wall structure such as chitin, lipids and peptides which may complicate the extraction of nucleic acids (RODRIGUES et al., 2017).

Knowing the limitations of fungal cell lysis, it is essential to add steps to the process, such as enzymatic digestion or the use of chemicals, mechanical interruption or sonication (ALAEY et al., 2005). With these added steps, DNA extraction protocols become extensive and laborious procedures to achieve good quality DNA (CENIS, 1992). In addition, several extraction steps can increase the risk of contamination and cause subsequent problems, such as the amplification of the material by PCR, therefore, they must be considered as an important selection criterion (ROMANELLI et al., 2014). Basically, the microbial DNA extraction process consists of physical, chemical and/or enzymatic cellular dissolution of cells and purification of the material

(TABATABAEI et al., 2010). The lysis procedure should be performed in such a way that it can extract as much DNA as possible without impurities (MAZZIOTTI et al., 2017). Commercial extraction kits can sometimes be inefficient since they are not able to account for individual needs of the sample, and they are relatively expensive (KUHN et al., 2017).

The search for an adequate protocol needs to evaluate yield, handling, cost, equipment dependence, reagent availability and toxicity to the environment and to the user (RODRIGUES et al., 2017). The extraction quality of the sample may present different results according to the procedure used (KUHN et al., 2017), so, the present work proposes to compare five DNA extraction procedures applied to filamentous fungal samples in relation to the purity, extraction yield and PCR quality of the extracted DNA.

11.4 MATERIAL AND METHODS

Three samples of the filamentous fungal genus *Penicillium* (strains R30, S39, N96), one sample of the genus *Allophoma* (S45) and another of the genus *Byssochlamys* (N102) were collected from the collection of microorganisms from the LEPETRO - Excelência em Geoquímica Petróleo, Energia e Meio Ambiente of the Universidade Federal da Bahia in Brazil with the deposit number BR1020210023414. The samples were initially obtained for the experiment by Lima et al. (2017) and reactivated by the method of peal of mycelium cubes (0.5 cm Ø) and inoculated in Petri dishes containing Sabouraud Dextrose agar (Liofilchem®, Italy) and 0.1 $g.L^{-1}$ Chloramphenicol (Vetec®, Brazil). They were then incubated for 7 days at 30 °C.

The fungal strains were submitted to five extraction methods (A, B, C, D and E). Initially two discs of mycelium were removed from each sample to obtain the DNA extract and treated according to the methods described below. These protocols were selected due to their different mechanisms used for cell lysis and because they span a variety of types: a commercial kit, a commercial kit with automatic extraction, a routinely used manual kit, and a manual kit where the reagents can be derived from materials commonly found in a molecular biology laboratory.

Method A was performed using the Fungi/Yeast genomic DNA isolation kit (Norgen Bioteck®, Canada) following the manufacturer's protocol and further subjected to ultrasound at 130 kHz per approximately every 8 min after step C of the protocol. The kit uses silica beads and buffer solution without phenol or chloroform in its composition.

Method B1 was performed with the Maxwell® 16 LEV simply-RNA cell kit (Promega®, United States). Cell lysis was initiated by manual maceration of the strains in 100 µL of ultrapure water (LGC technology®, Brazil). The extracts were then added to the cartridge provided by the kit and processed into Maxwell® 16 equipment following the manufacturer's instructions. The Method B2 was performed in the same way but with the addition of 10 µL of proteinase K (Invitrogen®, United States) and then incubated in a bath at 65 °C for 1 h.

Method C was performed with the same commercial kit of method A, according to protocol and addition of pretreatment. Initially the cell breakdown was performed by adding 500 µL lysis buffer composed of 1 $mol.L^{-1}$ NaCl, Tris pH 7.5 (Vetec Química®, Brazil), 0.5 $mol.L^{-1}$ EDTA (Chemco®, Brazil) and 20 µl (20 $ng.mL^{-1}$) proteinase K. The samples were ub sequently agitated for 1 min and 30 s with beads of silica supplied by the kit, followed by heating at 60 °C for 12 h. The extract was shaken for 2 min and 30 s twice before proceeding with the manufacturer's instructions.

Method D was performed with the same initial procedure from the previous step followed by the method described by Chomczynski and Sachi (1987). After incubation at ± 60 °C for 12 h, 400 µL (4 $mol.L^{-1}$) guanidine thiocyanate (Ludwig Biotecnologia®, Brazil), 100 µL of Brazol® (LGC Technology®, Brazil) and 2 iron beads were added. Then the tubes were shaken for 5 min and then the DNA was extracted with the addition of 250 µL chloroform (Dinâmica Química®, Brazil). After centrifugation at 12000 rpm for 20 min, 500 µL pure ethanol was added and centrifuged for 15 min. The supernatant was discarded and 500 µL 80 % ethanol was added. After centrifugation for 10 min and drying, the pellets were resuspended in 100 µL of TE buffer (LGC Technology®, Brazil).

Method E was in accordance with the modified protocol of Ivanova et al. (2006). Initially the strains underwent the pre-treatment according to method C. Then, 100 µL of lysis buffer was added and samples were incubated at 60 °C for 12 h. The extract was centrifuged at 12000 rpm for 1 min and 100 µL of Binding mix buffer added. After centrifugation at 14000 rpm for 2 min, 180 µL of centrifuged Protein wash buffer was added, and 750 µL of Wash buffer was added and centrifuged again. The pellet was washed with 80 % ethanol and centrifuged at 12000 rpm for 2 min. Subsequently, the residual ethanol was removed, and the pellet was resuspended in 50 µL of TE. The main characteristics of the methods used as well as their references thereof are listed in Table 4.

Table 4 - Summary of the characteristics of five methods of extracting DNA from filamentous fungi

Methods	References	Cell lysis	DNA extraction
A	Fungi/Yeast genomic DNA Isolation Kit (Norgen® Biotek, Cat. 27300)	Ultrasound /Beads	≠ formalin or chloroform
B1	Maxwell® 16 LEV simply-RNA cell (Promega® - Cat. AS1270)	Maceration	Guanidine Thiocyanate and Guanidine Hydrochloride
B2	Maxwell® 16 LEV simply-RNA cell (Promega - Cat. AS1270)	Maceration /proteinase K	Guanidine Thiocyanate and Guanidine Hydrochloride
C	Fungi/Yeast genomic DNA Isolation Kit (Norgen® Biotek, Cat. 27300)	Maceration /proteinase K/Beads	Tris:EDTA and other ≠ formolin or cloroform
D	Chomczynski e Sachi (1987)	Maceration /proteinase K/Beads	Tris:EDTA/Phenol/Guanidine
E	Ivanova, de Waard e Hebert (2006)	Maceration /proteinase K/Beads	Tris:HCl-EDTA/Guanidine

Source: Author, 2022.

The total DNA concentration was determined through two methods: by fluorescence and spectrophotometry. The fluorescence was measured in Quantus®. The DNA was subjected to dilution according to the manufacturer's protocol, where 1 μL of the sample is mixed with 199 μL of the ONE sDNA® reagent, incubated for 5 min in the dark and then read in the range 510-580 nm. The absorbancewas measured at 260 nm using the NanoDrop® Lite spectrophotometer. The integrity of the extracted DNA was visualized on 2 % (v/v) agarose gel (Life tecnologia®, Brazil) with subsequent visualization under UV light by Loccus Biotechnology's L-Pix transilluminator®. Electrophoresis was performed for 40 min at 100 volts in TBE buffer (pH 8.0). The protocol adopted was 5.0 μL of (0.4 %) bromophenol blue (Vetec Química®, Brazil), 4.0 μL of the sample and 1.0 μL of GelRed® (Biotium, Canada).

After the extraction of genomic DNA, the quality of the genetic material was confirmed by PCR (Polymerase Chain Reaction). The nuclear ribosomal internal transcript spacer (ITS) region was used as the fungal barcode. Method A amplifications were performed in volumes of 10 μL containing 2 μL of target DNA (>20 $ng.\mu L^{-1}$), consisting of 5.55 μL of 10 xTop Taq® PCR buffer (containing 15 mM $MgCl_2$), 1.0 μL of each primer (0.5 μM) and 0.45 μL of ultrapure water. The primers used were the ITS4 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS5 (5'-TCCTCCGCTTATTGATATGC-3') (SCHOCH et al., 2012). The PCR amplification was performed in an MJ Research PTC100® thermocycler and was preceded by a 5 -min denaturation at 94 °C, followed by 35 cycles of 30 s at 94 °C, 45 s at 53 °C and 60 s at 72 °C. The amplification ended with an elongation step of 5 -min at 72 °C. The PCR products were visualized by 2 % agarose gel electrophoresis (Figure 22).

The results were subjected to statistical analysis using the RStudio (version 3.4.2, Rcmdr and Psych packages). The parameters analysed were normality and homogeneity, using a check of variance.

11.5 RESULTS AND DISCUSSION

Table 5 and 6 show the concentrations of total DNA extracted from filamentous fungi according to the protocols tested. It was observed that through the statistical evaluation there were no differences ($p > 0.05$) between the protocols, however we can take into consideration other factors. It was observed that the extraction methods B1 and D obtained higher concentrations of DNA by fluorometric quantification and B1 and B2 for spectrophotometry. On the other hand, method C was the least efficient in both. The mean values of Quantus® ranged from 0.56 to 4.94 $ng.\mu L^{-1}$ while those of NanoDrop® ranged from 19.78 to 170.57 $ng.\mu L^{-1}$.

Knowing the analytical specificity of devices, we can analyse which method is best suited, considering the fact that the preanalytical quality of the samples will directly influence the diagnosis (CARNEVALE, 2015). Therefore, the quantity of samples was verified by the NanoDrop® equipment in parallel to Quantus®. The NanoDrop® is a spectrophotometer that uses absorbance at 260 nm to quantify and qualify nucleic acids (THERMO SCIENTIFIC, 2012). On the other hand, Quantus® is a fluorimeter that has the ability to quantify DNA at concentrations ≥ 10 pg in 1 μL of sample. Its mechanism uses a fluorescent dye that binds to DNA and provides it with 200-fold greater sensitivity than NanoDrop® in the reading of dsDNA (PROMEGA, 2017). In this sense, the variations in values detected between the equipment are justified by the difference in the detection mechanism of data performed by the equipment. Although NanoDrop® has provided higher values in all protocols, it proved to be questionable based on the sensitivity of Quantus®. It is believed that the samples may contain impurities that may have been detected by the spectrophotometer however, they can be refuted or confirmed by evaluating the purity and integrity of the samples. The amount of DNA required varies according to the molecular technique to be used later (COSTA; MOURA, 2001). Values > 20 $ng.\mu L^{-1}$ of DNA can be considered essential for amplification in PCR however, samples with lower concentrations and high quality can also generate satisfactory results (ROMANELLI et al., 2014). In this sense, procedure A stands out in relation to the others. This fact can be supported by the study by Barbosa and contributors (2018) where the use of the NORGEN® kit allowed the

obtaining of satisfactory products for amplification (ITS) and sequencing of filamentous fungi. The differences detected between the strains were expected since the unique characteristics of each species can influence the DNA extraction process. For this reason, the assessment of the general average and standard deviation of the data was taken into account, and thus Method A excels with more homogeneous results and higher quantity simultaneously.

Table 5 - Yield of total DNA samples determined with spectrophotometer in ng.µL

Methods	DNA sample (ng.µL^{-1}) Nanodrop® Spectrophotometer						
	R30	**S39**	**S45**	**N96**	**N102**	**General average**	**Standard deviation**
A	107.50	30.00	49.80	37.85	33.80	51.79	22,28
B1	97.45	138.05	20.20	255.85	341.30	170.57	102,40
B2	184.60	14.50	23.70	41.55	182.55	89.38	75,35
C	4.65	-1.35	45.25	14.45	35.90	19.78	16,63
D	75.45	62.15	18.05	31.70	46.30	46.73	17,65
E	17.65	33.45	45.20	14.45	40.25	30.20	11,32

Source: Author, 2022.

Table 6 - Yield of total DNA samples determined with fluorescence in ng µL

Methods	DNA sample (ng.µL^{-1}) Quantus® Fluorimeter						
	R30	**S39**	**S45**	**N96**	**N102**	**General average**	**Standard deviation**
A	3.37	0.91	1.95	2.24	1.37	1.96	0,66
B1	3.98	0.83	0.53	8.40	11.0	4.94	3,80
B2	3.52	0.33	0.64	0.96	6.20	2.33	2,02
C	0.42	0.57	0.75	0.49	0.60	0.56	0,08
D	4.10	5.40	1.54	1.93	2.80	3.15	1,27
E	1.10	1.27	1.77	1.55	1.24	1.38	0,21

Source: Author, 2022.

All samples were quantified by concentration and the DNA quality by spectrophotometry. The results referring to quality can be seen in Table 7. The purity was lower than the desired purity in most samples. Method A it was the only one that presented adequate degree of purity for all samples with an general average of 1.95 ng.µL^{-1}, however it presented a low concentration of the extracted material. On the other hand, it was found that the method B showed the best results in the amount of DNA extracted, with a high protein concentration.

With regard to the quality of the samples, the wavelength $\lambda = 260$ nm is used to measure the DNA concentration and from the absorbance reading of A260/A280 nanometers, the purity of the sample can be estimated (TRIPATHY et al., 2017). Samples are considered contaminant free when the A260/A280 ratio values are between 1.8 to 2.0 (LUCENA-AGUILAR et al., 2016).

In the case of method A, the quantity may not be a problem since, for the purposes of PCR-based assays, the sample yield can be evaluated with reduced significance, including very small amounts of DNA (ROMANELLI et al., 2014). Since the mentioned method used sonication, the purity results are in accordance with the literature since the use of ultrasonic waves for cell lysis results in high product purity due to selectivity in materials released and is thus considered a very efficient and reliable tool (TRIPATHY et al., 2017). To increase the yield of method B samples, it is possible to suggest modifications in the protocol, as well as in the study by Kumar and Mugunthan (2018), which extracted DNA from fungal strains using the NORGEN® kit with the addition of proteinase K and sonication with beads.

Table 7 - Purity of the total DNA samples determined by the ratio 260/280 quantified in Spectrophotometer

	A_{260}/A_{280} samples ($ng.\mu L^{-1}$)					
Methods	**R30**	**S39**	**S45**	**N96**	**N102**	**General average**
A	2.02	1.84	2.02	1.92	1.96	1.95
B1	1.72	1.29	1.38	1.48	1.90	1.55
B2	1.70	0.97	1.48	1.23	1.75	1.42
C	0.57	0.79	1.62	0.49	1.17	0.92
D	1.68	1.20	0.99	1.16	1.10	1.22
E	1.10	0.71	0.75	0.66	0.65	0.55

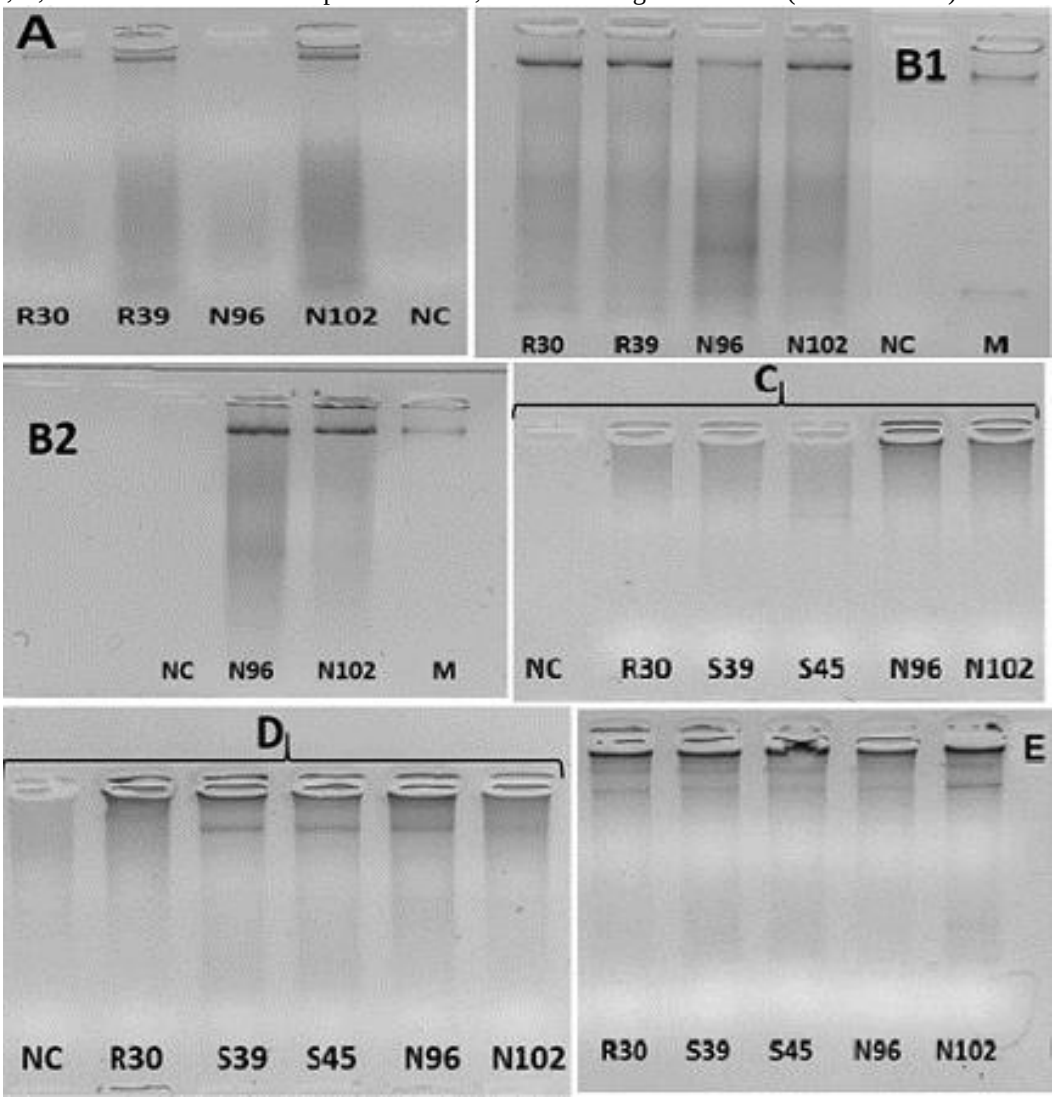
Source: Author, 2022.

Although the signals of the DNA band profiles visualized on the gel were weak, it was possible to observe that there was a difference between the protocols tested according to Figure 21. Protocols A and B showed band profiles for most samples with quality that can be considered regular. On the other hand, no nucleotide band profile was observed for the fungal isolates with extraction protocols C, D and E, except sample R30 for method D. Thus, the procedures used in C, D and E they proved to be unsatisfactory for this application.

While spectrometry generates numerical values, electrophoresis is a visual assessment, but is equally important. The use of the gel allows observation of the integrity and impurity of the nucleotide samples. In this way, both techniques complement and expose more robust data (CORDEIRO et al., 2017). Since it was possible to visualize consistent patterns of DNA bands in electrophoresis, the possibility of a false positive and degraded sample was ruled out. The absence or little apparent sign of the bands on the gel corroborates the results shown in Table 5 and 6 and confirms that the values displayed on the NanoDrop® Spectrophotometer are underestimated. The absence of bands was probably due to a combination of the small amount of

sample used for analysis and the protocol's inability to extract DNA from a variety of fungal species. Protocols A and C were executed with the same kit. However, it was evident that the changes suggested in C were not efficient. According to Smit et al. (1999) the homogenization of fungal DNA with the aid of glass beads or similar matrix for cell lysis can result in high quality DNA for PCR amplification. However, method A, which used ultrasound, obtained better results than maceration and vortexing with beads. Most likely, the time of less than 30 min of homogenization with beads was not enough to extract adequate amounts of DNA from the culture of filamentous fungi as acquired in Burik and contributors (1998).

Figure 21 - Agarose gel electrophoresis (2 %) of the total DNA extracted from the filamentous fungi using methods A, B1, B2, C, D and E. Lane M = 100 bp DNA ladder, Lane NC - negative control (distilled water).



Source: Author, 2022.

To assist in deciding which technique is most advantageous, we can combine yield information with time spent and exposure to solvents. Table 8 shows the procedures used in C, D and E form the longest lasted more than 700 min.

Manual extraction methods are generally cheaper than commercial kits however, the use of phenol chloroform, as well as in method D, can be considered outdated for a cost benefit. In addition to being time consuming, they may not produce the expected results and may expose the researcher to toxic chemicals, which should be avoided in routine practice (KUMAR; MUGUNTHAN, 2018). Rodrigues and contributors (2017) state that it is possible to obtain high-yield, pure DNA from fungal material without using strong cell disruption procedures or toxic reagents such as phenol or mercaptoethanol and without increasing the cost of the process. In this sense, the possibility of using protocols C, D and E to extract the samples in question can be ruled out. Although method B is faster and automation can reduce the possibility of technical error, it is more suitable for tissue samples according to a study by Jeffries and contributors (2014). The Maxwell® kit requires specific and expensive equipment, showing the need to add prerupture and/or cleaning steps, increasing the cost of the process. In addition, the extraction process in closed system equipment does not allow adjustments to the steps, disregarding the variability of the samples. Thus, it was concluded that method B is not ideal. According to the data in Table 4, method A showed the best results since the average quality values were within the ideal range, it is easy to use, it did not involve handling harmful reagents, and is relatively inexpensive compared to method B.

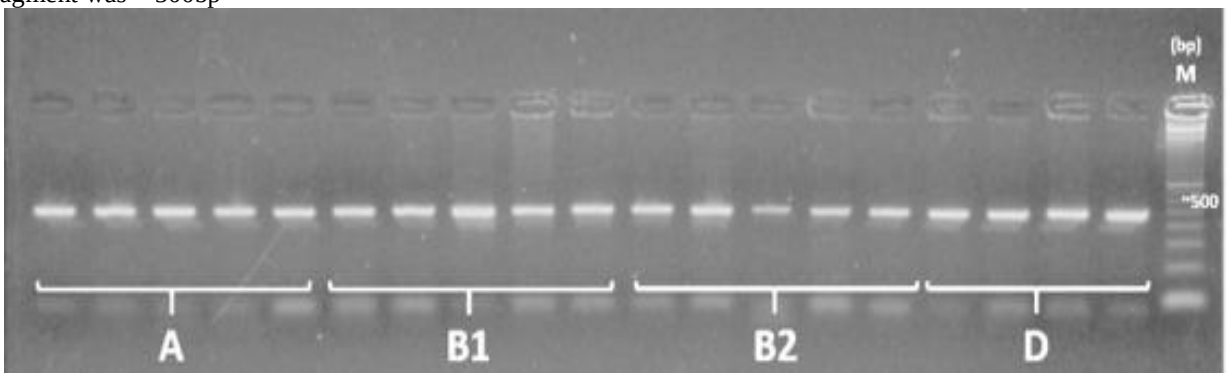
Table 8 - An overview of the characteristics of DNA extraction from filamentous fungal samples

Methods	**Time (min)**	**Noxiousness**	**General average Quantity**		**Average Quality**
			Quantus®	Nanodrop®	
A	40	No toxic	1.96	51.79	1.95
B1	50	Harmful	4.94	170.57	1.55
B2	120	Harmful	2.33	89.38	1.42
C	770	Harmful	0.56	19.78	0.92
D	775	Toxic	3.15	46.73	1.22
E	740	Harmful	1.38	30.20	0.55

Source: Author, 2022.

The results of the confirmatory PCR of DNA extraction using the ITS gene can be seen in Figure 22, indicating that method A presented the best bands for amplifying the DNA fragment (~500 bp), when compared with the other analyzed methods.

Figure 22 - Agarose gel electrophoresis of amplicons obtained by PCR amplification of total DNA (ITS gene) from the filamentous fungi using methods A, B1, B2 and D. Lane M = 100 bp DNA ladder. The size of the ITS gene fragment was ~ 500bp



Source: Author, 2022.

11.6 CONCLUSION

Among the protocols described, Method A was a simple, relatively fast alternative with satisfactory DNA yield and purity. For the other protocols, it is necessary to make adjustments in order to improve the performance of the steps, considering the facility and cost of the process as a whole. Thus, our data suggest that the protocol using the Norgen® isolation kit can provide regular amounts and concentrations of good quality DNA for applications in molecular techniques. The method is technically easy and does not require toxic chemicals, thus, it is safe for operators and the environment, and is not dependent on specific and expensive equipment. In addition, the amplification of the fragment of the ITS gene allowed us to confirm the quality of the extracted DNA, since the reaction did not produce dimers or nonspecific amplification.

7 BIOTECHNOLOGICAL INTEREST MICROORGANISMS MOLECULAR IDENTIFICATION

7.1 ABSTRACT

Classic microbiological identification techniques, although important for understanding isolated microorganisms, do not provide complete information about the diversity, richness, and abundance of the microbiological community. On the other hand, molecular biology techniques such as DNA Barcode make it easier for the identification of microorganisms not to be done unequivocally. The aim of this study is to molecularly identify, using the DNA Barcode technique, fungi and bacteria that are of biotechnological interest. 25 fungal and nine bacterial isolates that constitute a mixed microbial consortium were identified using the ITS and 16S genes, respectively. Six species of fungi distributed in four genera were detected, while for bacteria five species and four different genera were observed. The molecular biology tools helped to identify the microorganisms that make up the consortium and corroborated through genetic evidence the potential to remediate areas impacted by petroleum-derived hydrocarbons. The *Allophoma* fungus genus and the *Brevibacillus* bacteria genus are not commonly associated with this process, opening paths to obtain new possible bioproducts and new studies to better understand the bioremediation process of these microorganisms.

Keyword: bioremediation, biodegradation, organic compounds, environmental biotechnology, marine microorganisms

7.2 INTRODUCTION

Contamination by petroleum-derived hydrocarbons affects numerous global sites, largely due to the activities of the petroleum industry, released industrial waste, anthropogenic activities, and accidental spills, which are factors that currently raise valid environmental concerns (GKOREZIS et al., 2016). Thus, it is necessary to develop effective methods for remediating areas impacted by petroleum-derived hydrocarbons. In this context that biotechnology or, more precisely, bioremediation can serve as a tool for protecting the environment and humans from xenobiotic contaminants (RAGHUNANDAN et al., 2018). When compared to other techniques, such as physical and chemical remediation, the advantages of bioremediation are its low cost, relatively simple operation and minimal disturbance to the soil (VELEZ et al., 2017).

The characterization of the microbiota involved in the bioremediation process of areas impacted by hydrocarbons is vital to the ultimate success of such endeavours, since the biodegradation of oil and other hydrocarbons in natural environments is a complex process, with quantitative and qualitative aspects that depend on the nature and amount of oil or hydrocarbons

present in the environment, seasonal conditions, and the abundance, diversity, and structure of the autochthonous microbial community (SANDRIN et al., 2003; VAN HAMME et al., 2003; PAL et al., 2017). The use of microorganisms, especially fungi and bacteria, for bioremediation processes has been routinely reported in recent years, due to the enormous genetic potential of specific organisms and their adaptability for biochemically cleansing contaminated environments (FUENTES et al., 2014; ZAFRA et al., 2016).

The use of mixed communities of microorganisms, also called mixed microbial consortia, for the remediation of areas impacted by complex residues or toxic chemical pollutants more predictably promotes satisfactory results compared to the use of single microbial strains (WAGHMODE et al., 2011). The bioremediation process is enhanced by microbial consortia because the biochemical capabilities of multiple, networked microorganisms can collectively mediate a greater variety of metabolic biodegradation functions (ZUROFF; XIQUES; CURTIS, 2013). Studies in the literature have already demonstrated the effectiveness of microbial consortia as a bioremediation tool for areas impacted by petroleum-derived hydrocarbons (EBADI, et al., 2017; PUGAZHENDI et al. 2017; XIA et al., 2019; CUI et al., 2020; KOOLIVAND et al., 2020).

Classic microbiological identification techniques based on cell cultures, although particularly important for understanding aspects related to the physiology and taxonomy of isolated microorganisms, do not provide complete information on the diversity, richness, and comprehensive metabolic capabilities of microbiological communities (VAN HAMME et al., 2003). Alternatively, the identification of genera and species of fungi and bacteria using molecular biology techniques, including the sequencing of taxonomically relevant genes, makes it more straightforward to identify specific microbial consortia that possess suitable metabolic capabilities for remediation (CHAKRABORTY et al., 2014). The bioremediation process is enhanced by microbial consortia because the biochemical capabilities of multiple, networked microorganisms can collectively mediate a greater variety of metabolic biodegradation functions (HERBERT et al, 2003). For the identification of bacteria, the sequencing uses genes such as 16S rRNA and rpoB is used, while for fungi, 28S (LSU) and ITS genes (CASE et al., 2007; SCHOCH et al., 2012). The bioremediation process is enhanced by microbial consortia because the biochemical capabilities of multiple, networked microorganisms can collectively mediate a greater variety of metabolic biodegradation functions (GHOSH; CHOWDHURY; BHATTACHARYA, 2016).

The objective of this study was to use the DNA barcode technique to molecularly identify fungi and bacteria that are of biotechnological interest for remediation of areas impacted by petroleum hydrocarbons, the production of undesirable bioproducts, and various industrial wastes.

7.3 MATERIAL AND METHODS

Twenty-five filamentous fungi (R2, R11, R16, R26, R27, R28, R30, R31, R33, S36, S38, S39, S40, S41, S45, S52, A79, A80, A83, A84, N82, N89, N96, N101, and N102) and 9 bacteria (PD6, RB4, RC6, PD8, RA2, RC5, PD7, PD5, and OH4) were isolated from mangrove sediment samples, located in the northern portion of the Todos os Santos Bay, near the Rio São Paulo (LIMA et al., 2018), all of which are of biotechnological interest because they possess the genetic capacity to degrade petroleum-derived hydrocarbons and the potential for comprising a mixed microbial consortium for hydrocarbon bioremediation, protected by patent with deposit number BR 10 2021 002341 4 at INPI (National Institute of Industrial Property). The individual isolates are stored in the microorganism bank of the Excelência em Geoquímica Petróleo, Energia e Meio Ambiente (LEPETRO), located at the Instituto de Geociências (IGEO) of the Universidade Federal da Bahia (UFBA).

For the extraction of total DNA from bacterial samples, the Symple RNA Purification kit was used for automated DNA or RNA extraction using Maxwell® 16 (Promega) equipment in the LEV (low elution volume) configuration and following the manufacturer's instructions. For fungal samples, the Genomic DNA Isolation kit (Norgen Biotek Corp.) was used with the aid of a bead disruptor for mechanical lysis. The extracted DNA was resuspended in 50 µL of ultrapure water without DNAse.

The quantification of the total extracted DNA was conducted using two methods: first, a 1 % agarose gel was formed, the samples were stained with bromophenol blue and SYBR Gold® (Thermo Fisher) and subjected to electrophoresis paired with a standard sample (lambda) of 25 ng of DNA per µL. Finally, the total DNA was visualized in a transluminator. In addition to this method, the samples were analyzed in a Nanodrop One® (Thermo Fisher) spectrophotometer to measure the quality and purity of the extracted DNA.

PCRs for amplification of the ITS region were conducted using the following protocol: 9.0 µL of TopTaq Mastermix® (Qiagem), 1.3 µL of ultrapure H_20, 0.2 µL of ITS4 primer (10

pmol) – TCCTCCGCTTATTGATATGC (WHITE et al., 1990), 0.2 µL of the ITS5 primer (10 pmol) – GGAAGTAAAAGTCGTAACAAGG (WHITE et al., 1990), 3.0 µL of the TBT additive – trehalose, BSA and Tween-20 (SAMARAKOON et al., 2013), 0.3 µL of the DMSO aditive, and 1 µL (40 ng) of total DNA, totalling a reaction mixture of 15.0µL. Amplification was conducted in a thermocycler (SureCycler 8800 Thermal Cycler - Agilent) using the following program: a denaturation cycle at 94 °C lasting 5 minutes, followed by 35 cycles of: denaturation at 94 °C for 45 seconds, annealing for 45 seconds at 55 °C and extension for 1 minute at 72 °C and a final extension cycle at 72 °C for 7 minutes.

For the amplification of the 16S rRNA region, the following protocol was performed: 9.0 µL of TopTaq Mastermix® (Qiagem), 1.3 µL of ultrapure H_20, 0.2 µL of primer V4 (10 pmol) – CCTACGGGRSGCAGCAG (WANG; QIAN, 2009) , 0.2 µL of the V5 primer (10 pmol) – CTTGTGCGGGCCCCCGTCAATTC (WANG; QIAN, 2009), 3.0 µL of the TBT additive – Trehalose, BSA and Tween-20 (SAMARAKOON et al., 2013), 0.3 µL of the DMSO additive and 1µL (40 ng) of total DNA, totaling the reaction in 15.0 µL. Amplification was carried out in a thermocycler (SureCycler 8800 Thermal Cycler - Agilent) using the program: a denaturation cycle at 95 °C lasting 10 minutes, followed by 30 cycles of: denaturation at 95 °C for 40 seconds, annealing for 40 seconds at 55 °C and extension for 1 minute at 72 °C and a final extension cycle at 72 °C for 10 minutes.

The amplified fragments were subjected to electrophoresis in a 2 % agarose gel and stained with bromophenol blue and SYBR Gold® (Thermo Fisher). In the electrophoresis run, a molecular weight marker of 100 base pairs (bp) ladder (GE Healthcare®) was added and, finally, the fragments were visualized in a transluminator, after which they were later photographed, and the photos filed.

The products of the partial amplification of the ITS and 16S genes were purified using the 20 % Polyethylene Glycol (PEG) DNA precipitation protocol. The sequencing reactions were performed by the direct method, in both directions - forward and reverse - containing: 50 ng of purified PCR product, 1.0 µL of sequencing buffer (Save Money 5X), 0.5 µL of BigDye v 3.1 (Applied Biosystems®), 0.25 µL of the primer (3 pmol) and an amount of ultra pure water that yielded a total volume of 10 µL. Following the sequencing reaction, which consisted of 35 cycles consisting of a step at 96 °C for 15 s, a step at 52 °C for 10 s and a polymerization step at 60 °C for 4 min, the samples were kept at 4 °C for analysis. Then, 80 µL of isopropanol was added to

each sample. After 15 min at room temperature, the samples were centrifuged at 4,000 rpm in a refrigerated tube centrifuge for 25 min at 4 °C. After DNA precipitation, the supernatant was discarded and the samples were washed twice with 150 μL of 70 % ethanol followed by centrifugation at 4,000 rpm for 15 min at 4 °C. The samples were dried at room temperature or in a thermocycler (50 °C for 15 min), resuspended in 10 μ L of formamide, denatured for 5 minutes at 95 °C and submitted to sequencing in an automatic sequencer 3500 DNA Analyzer sequencer according to the methodology suggested by the manufacturer of the equipment (Applied Biosystems®).

Forward and reverse sequences of each sample were compared, corrected and edited in the BioEdit program (HALL, 1999), creating a single consensus sequence and aligned using the MEGA X program: Molecular Evolutionary Genetics Analysis across computing platforms (KUMAR et al., 2018). The search for sequences similar to those obtained in this work was performed using the BLAST program (ALTSCHUL et al., 1990) in the NCBI GenBank™ database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). These sequences were deposited at the NCBI GenBank™ at the end of the work.

The evolutionary relationship of taxa was constructed by the UPGMA method (SNEATH; SOKAL, 1973) using a matrix of characters with all taxa, using the K2P model and applying 10000 bootstrap replicates to obtain cluster support (FELSENSTEIN, 1985).

7.4 RESULTS AND DISCUSSION

For fungal isolates, four genera and six species were identified (Table 9), while for bacterial isolates, the identification showed four genera and five species (Table 10). The epithets of the species were suppressed due to the protection of these strains by patent number BR 10 2021 002341 4, as they make up a proprietary, the mixed microbial consortium with the capacity for degrading petroleum-derived hydrocarbons.

The fungal dendrogram (Figure 23) showed that isolates of the same species were grouped together, except R33, which was grouped together with *Penicillium* species however, this isolate is located on the same branch with all other isolates of the same species. *Allophoma* sp.1 and *Byssochlamys* sp.1 occupied a branch as to all other isolates, indicating phylogenetic divergence in relation to the genera *Aspergillus* and *Penicillium.* The phylogenetic tree of the bacterial isolates (Figure 24) separated all species clearly, evidencing a phylogenetic proximity

between the genera *Bacillus* and *Brevibacillus* and between *Pseudomonas* and *Stenotrophomonas*. The two species of *Pseudomonas* were grouped in the same branch..

Table 9 - Molecular identification of fungal isolates and their respective access numbers

Sample Code	Access Number (NCBI*)	Closest Relative Species	DNA sequence similarity (%)
R2	MW865711	*Aspergillus* sp.1	100
R11	MW855898	*Penicillium* sp.1	100
R16	MW855899	*Aspergillus* sp.1	100
R26	MW855900	*Penicillium* sp.1	100
R27	MW865712	*Penicillium* sp.1	100
R28	MW865713	*Penicillium* sp.1	100
R30	MW865714	*Penicillium* sp.1	100
R31	MW855901	*Penicillium* sp.2	100
R33	MW865715	*Aspergillus* sp.2	100
S36	MW865716	*Penicillium* sp.1	100
S38	MW865717	*Penicillium* sp.1	100
S39	MW865718	*Penicillium* sp.1	100
S40	MW855902	*Penicillium* sp.1	100
S41	MW865719	*Penicillium* sp.1	100
S45	MW865720	*Allophoma* sp.1	100
S52	MW865721	*Penicillium* sp.1	100
A79	MW865722	*Penicillium* sp.2	100
A80	MW865723	*Penicillium* sp.1	100
A83	MW865724	*Penicillium* sp.1	100
A84	MW865725	*Penicillium* sp.2	100
N82	MW865726	*Penicillium* sp.2	100
N89	MW855903	*Byssochlamys* sp.1	100
N96	MW865727	*Penicillium* sp.2	100
N101	MW865728	*Byssochlamys* sp.1	100
N102	MW865729	*Penicillium* sp.1	100

* National Center for Biotechnology Information
Source: Author, 2022.

Tabela 10 - Molecular identification of bacterial isolates and their respective access numbers

Sample Code	Access Number (NCBI*)	Closest Relative Species	DNA sequence similarity (%)
RA2	MW881196	*Pseudomonas* sp.1	100
RB4	MW881197	*Bacillus* sp.1	100
RC5	MW881198	*Brevibacillus* sp.1	100
RC6	MW881199	*Stenotrophomonas* sp.1	100
PD5	MW881200	*Stenotrophomonas* sp.1	100
PD6	MW881201	*Pseudomonas* sp.2	100
PD7	MW881202	*Pseudomonas* sp.2	100
PD8	MW881203	*Stenotrophomonas* sp.1	100
OH4	MW881204	*Bacillus* sp.1	100

* National Center for Biotechnology Information
Source: Author, 2022.

Isolates of the *Penicillium* and *Aspergillus* found in oil contaminated soil, were treated as potential hydrocarbon degrading microorganisms using the bioaugmentation technique as a tool

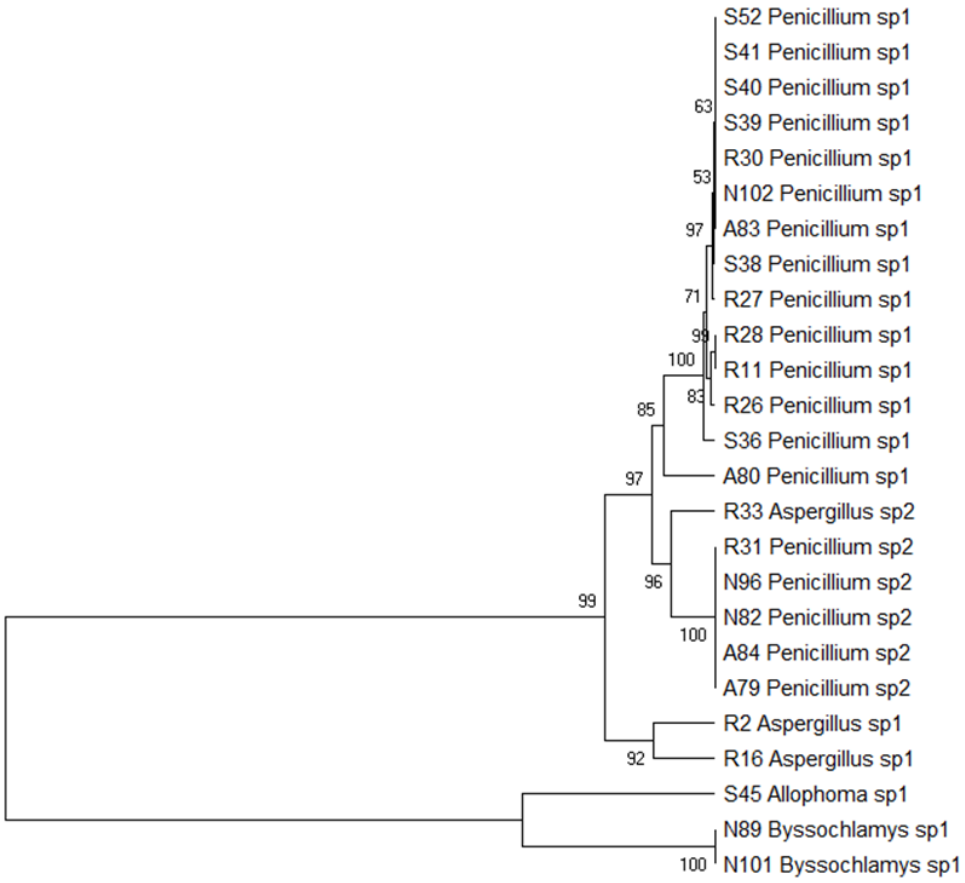
for bioremediation of these impacted environments and were molecularly identified by means of the DNA Barcode (SOKOLO et al., 2018). Fungi belonging to the genus *Penicillium* are typically good hydrocarbon assimilating organisms, and studies have indicated their ability to biotransform xenobiotic compounds into less toxic products (LEITÃO, 2009). Strains of the genus *Aspergillus* showed higher rates of biodegradation of crude oil than compared to fungi of other genera (JAWHARI, 2014; BURGHAL et al., 2016).

Krishnamoorthy et al. (2018) demonstrated the ability of microorganisms of the genus *Allophoma* (formerly known as *Phoma*) to decolour dyes, which can be applied specifically to the decolorization and degradation of azo dyes. This genus can also biodegrade of polychlorinated biphenyl (PCB), a compound widely used in the industrial processes (MOUHAMADOU et al., 2013). However, no attempts to use microorganisms of this genus for the biodegradation of petroleum-derived hydrocarbons were not found in the literature, indicating a biotechnological innovation for our mixed microbial consortium, which uses unconventional fungal strains to remediate areas impacted by hydrocarbons.

Fungal isolates of the genus *Byssochlamys* showed effective results in the bioremediation of soils impacted by pesticides, especially in mixed cultures, which did not generate other toxic compounds following the degradation process (HECHMI et al., 2016). Another strain of the genus showed considerable capacity for the degradation of high molecular weight PAHs (BOSSO et al., 2015). The "draft" genome of the BYSS01 isolate of *Byssochlamys* sp., isolated from jet fuel, found genes involved in the degradation of aromatic compounds and alkanes (RADWAN et al., 2018). Information such as this helps to better understand the adaptive mechanisms used by fungi to survive and proliferate in environments contaminated with a variety of hydrocarbons.

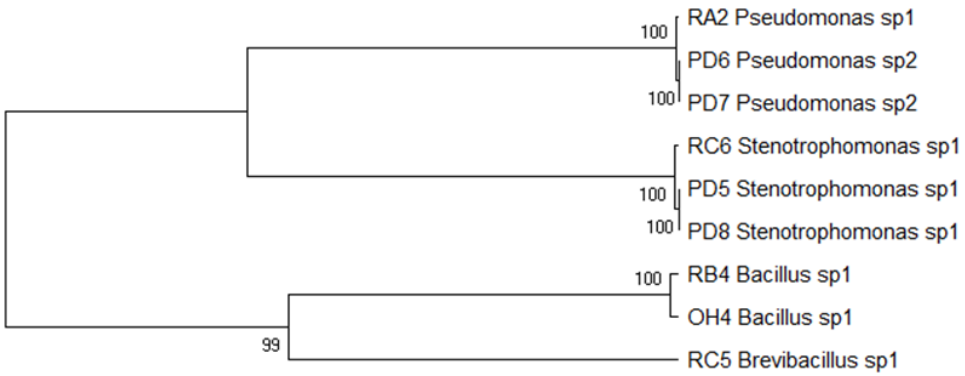
Bacterial isolates from the *Pseudomonas* and *Bacillus* genera were used to assess oil degradation through bioremediation (VINOTHINI et al., 2015). Cybulski et al. (2003) evaluated the production of natural emulsifiers by consortia of different species of these two bacterial genera, indicating that *Pseudomonas* species can play an important role during the hydrocarbon biodegradation process. In addition to bioremediating areas impacted by crude oil, mixed microbial consortia also have the potential to degrade phenolic compounds and hydrocarbons present in wastewater (SINGH et al., 2013).

Figure 23 - Phylogenetic tree using the UPGMA method based on data from the ITS gene of the fungal isolates from the mixed microbial consortium. Bootstrap values based on 10,000 repetitions are listed as percentages in the branches. Only bootstraps with values >50 % are shown



Source: Author, 2022.

Figure 24 - Phylogenetic tree using the UPGMA method based on 16S gene data from bacterial isolates from the mixed microbial consortium. Bootstrap values based on 10,000 repetitions are listed as percentages in the branches. Only bootstraps with values >5 0% are shown



Source: Author, 2022.

Pseudomonas is the most studied bacterial genus for use in the bioremediation of areas impacted by oil, and its potential to degrade petroleum-derived hydrocarbons has been corroborated in published reports (PACWA-PLOCINICZAK et al., 2014; KUMAR et al., 2014). Ojewumi et al. (2018) suggest that the use of a mixed microbial consortium formed by bacteria of the genus *Pseudomonas* and fungi of the genus *Aspergillus,* presents a greater capacity for the degradation and removal of crude oil when compared to these same microorganisms used alone.

Bacteria of the *Stenotrophomonas* genus have been described by Gosh and Saha (2013) for their promising potential to serve as effective biological agents in the removal of copper (Cu) from aqueous solutions, while Raman et al. (2017) showed their ability to remove chromium (Cr). Species of this genus have also been linked to the groundwater phytoremediation process due to their association with plant roots (MUKHERJEE; ROY, 2016). The inoculation of the *Stenotrophomona*s strain in oil refinery wastewater promoted the removal of compounds from the aromatic and saturated fractions of oil, as well as phenolic compounds, indicating the potential to bioremediate oil refinery effluents to reduce environmental impacts (PATEL; PATEL, 2020).

A strain of species from the genus *Brevibacillus* was used as a promoter of bioremediation of soils contaminated with arsenic (As) (Mallick et al., 2014). The cytochrome P450 monooxygenase gene was essential for the degradation of triphenyl phosphate (TPHP), generating metabolites that showed a negative correlation with TPHP residues when a *Brevebacillus* strain was used to promote the remediation process (WEI et al., 2018). Among all the genes of the P450 complex (CYP), the CYP52 family is linked to the first step in the assimilation of alkanes, while the genes belonging to the CYP53 family are associated with the oxidation of aromatic hydrocarbons (HUARTE-BONNET et al., 2017). The P450 genes are commonly associated with fungi, although some bacteria have these genes in their genome (NELSON, 2009), which is why, although not one of the most used genera for oil bioremediation processes, *Brevibacillus* has this potential, as shown in the experiment conducted by Phulpoto et al. (2017) with an isolate of this genus showing a strong potential for degradation of oil-based paint, which can also be used to remediate ecosystems polluted by other hydrocarbons.

7.5 CONCLUSION

The correct identification of microorganisms with biotechnological potential for use in bioremediation processes is essential to elucidate issues related to how these organisms may

perform during the biodegradation of xenobiotic compounds, which metabolic pathways are used, and the best bioremediation strategies to adopt. In this way, molecular biology tools, which helped in the identification of the microorganisms that comprise our mixed microbial consortium, provided the genetic evidence to corroborate their potential to degrade petroleum-derived hydrocarbons found in previous geochemical studies, in addition to illuminating a mixed community of genera that are not commonly associated with this process, including *Allophoma* and *Brevibacillus*.

This biotechnological innovation opens paths to study and obtain new bioproducts associated with the group of microorganisms that constitute the mixed microbial consortium that we identified. This study suggests a need for study of microorganisms that show taxonomic similarity to understand if they have a diversity of metabolic action that can further advance bioremediation processes.

8 MICROBIAL CONSORTIUM INTERACTION PERFORMANCE OF INTEREST FOR PETROLEUM DEGRADATION

8.1 ABSTRACT

Several microorganisms are important promoters of the bioremediation of environments. However, for them to be applied in a consortium, it is necessary to evaluate the interactions between species to avoid reducing or changing their functions. The objective of this study was to evaluate the interaction of fungi for the potential construction of a microbial consortium for oil biodegradation. Fungi of the genera *Aspergillus* sp, *Penicillium* sp. and *Byssochlamys* sp. were assessed with the compatibility test using the direct confrontation method for fifteen days at 30 °C. The results were evaluated by calculating the growth inhibition index, growth rate, resistance rate and classification of the type of interaction. The 21 combinations of fungi exhibited discoloration of the redox indicator, indicating the degradation of oil regardless of the type of interaction. The R16, N89 and A79 strains exhibited antagonistic activity; therefore, they should be considered for preferential individual use in degradation processes. On the other hand, the R26, R31 and S40 strains exhibited promising characteristics for use in mixture of microorganisms to promote efficient in petroleum mineralization. Thus, these strains have potential for application in the process of biodegradation process of petroleum hydrocarbons and/or its derivatives, as well as that of other persistent aromatic compounds.

Keywords: antagonism; bioremediation; filamentous fungi; microbial coculture; microbial synergism; mycoremediation

8.2 INTRODUCTION

The growing global demand for energy and industrial raw materials contributes to the extensive use of natural resources such as fossil fuels, depleting the resources and causing various disturbances in the environment, such as climate change and accidental oil spills (ZHANG et al. 2019). Although several remediation techniques are available, the restoration of environments contaminated by crude oil is still a constant challenge (VARJANI, 2017). To understand the scope and microbial interactions in a consortium, it is essential to understand the properties of crude oil and its effects in natural environments.

Crude oil consists of a complex mixture of compounds, mainly containing hydrocarbons and small quantities of other elements, such as sulfur, oxygen and nitrogen (SAMA et al., 2018). Among these compounds are recalcitrant, classified as priority pollutants because they are resistant to degradation due to their low reactivity (VARJANI, 2017). These compounds can be degraded by a variety of microorganisms and each species will act differently in the breakdown

of certain oil compounds (ZANAROLI et al., 2010). Through their complex interactions, mixed populations of microorganisms, through their complex interactions, have the ability to degrade molecules of higher molecular weight, such as polycyclic aromatic hydrocarbons (PAHs), thus demonstrating the great importance of applying bioaugmentation for the remediation of contaminated environments (SUN et al., 2010). The low biodiversity of the natural microbiota at the contamination site or their insufficiency of the organisms with regard to the specificity of the complementary substrate, which is important for the degradation of different hydrocarbons, can be a limiting factor for the bioremediation of areas impacted by oil contamination (RON; ROSENBERG, 2014). However, combining several microbial species in consortia, is advantageous for the oil mineralization process.

Microbial consortia are communities that have cooperative characteristics for degrading or metabolizing xenobiotic components (DESIDERATO et al., 2018). These consortia, usually formed by fungi and bacteria, carry out processes that the individual species would not be able to perform with the same efficiency (LIMA et al., 2018). Combining species results in greater robustness to environmental fluctuations, greater stability of strains over time, resistance to invasion by other species, and the division of functions. (CARREÑO; RESTREPO, 2010). The interaction among the microbial members ensures the coordination of functions for the decomposition of xenobiotics, expanding the functionality and maintenance of the homeostasis of the consortium (SUBASHCHANDRABOSE et al., 2011). The formation of specific consortia to perform certain functions is based on these interactions which can gradually change the composition of the community (JIMÉNEZ et al., 2018). The species in the associated communities, generally, establish synergistic relationships, intensifying the level of degradation of the required pollutant on the other hand, other dynamics can also affect the relationships in these consortia, such as competition, neutralism, antagonism, and mutualism (CORTES-TOLALPA et al., 2017).

Antagonism in consortia is caused by deleterious effects, mainly in the form of competition for spaces and nutrients, causing the inhibition and production of toxic metabolites (CANO et al., 2011). This type of interaction directly affects the efficiency of the desired functions of the consortia. Synergistic, the microbial interactions of associations allow exchanges of growth-stimulating compounds, and advantageously, the optimized use of their regulatory systems, making it possible to overcome the problems of metabolic repression and feedback

regulation which are common for single strains (CORTES-TOLALPA et al., 2016). Insights about microbial interactions can advance the environmental sustainability and economic viability of biological systems however, the understanding of their mechanisms of their interaction is still limited (NATRAH et al., 2014). To expand the possibilities of a consortium, it is necessary to create combinations of microbial strains suitable for the degradation process by considering the compatibility and interactions between species. The aim of this work is to evaluate the synergistic compatibility of a group of seven fungi endogenous to the Todos os Santos Bay in an in vitro assay for the construction of a microbial consortium to expand the potential for use as agents in the biodegradation of hydrocarbons from petroleum and/or its derivatives.

8.3 MATERIALS AND METHODS

The study was conducted at the Centro de Excelência em Geoquímica Ambiental e do Petróleo (LEPETRO) at the Universidade Federal da Bahia. Seven filamentous fungal strains denoted as: R11, R16, R26, R31, S40, A79 and N89 (*Penicillium* sp. 1, *Aspergillus* sp, *Penicillium* sp. 1, *Penicillium* sp. 2, *Penicillium* sp. 1, *Penicillium* sp. 2 and *Byssochlamys* sp.), from Bahia de Todos os Santos mangrove sediment near to the Landulpho Alves de Mataripe Refinery, were preselected from the studies described in Lima and contributors (2017) and used in this experiment. The microorganisms constitute a mixed microbial consortium (LIMA et al., 2021) protected by a patent at the National Institute of Industrial Property (INPI) with deposit number BR 10 2021 002341 4 and endorsed in the National Center for Biotechnology Information (NCBI) with the accession numbers MW855898, MW855899, MW855900, MW855901, MW855902, MW865722, and MW855903. Each fungal isolate was transferred from storage culture to Petri dishes containing Sabouraud dextrose agar (Kasvi®, Brazil) and incubated for 7 days at 30 °C. Subsequently, 1 cm diameter mycelium discs were cut from the edge of these cultures and used as an experimental inoculum. The strains were categorized according to their oil fraction degradation capacity as follows: 1 = saturated; 2 = aromatic; 3 = NSO compounds (nitrogen, sulfur, oxygen); 4 = saturated and NSO compounds; and 5 = saturated, aromatic and NSO compounds.

The antagonistic in vitro evaluation among the strains of fungi was performed using the method of cultures paired in Petri dishes. This method is based on the direct confrontation of

microorganisms positioned in a solid culture medium, for a predetermined period and subsequent analysis of the interactions between the tested strains (CORRÊA et al., 2007).

In Petri dishes measuring 9 cm in diameter, mycelium disks for each pair of combinations of fungal colonies were inoculated onto the agar surface, placed at equidistant points between strains and subsequently incubated at 30 °C in a germination chamber. Biochemical oxygen demand (BOD) in continuous darkness was assessed. The culture medium was prepared with Bushnell Haas (BH) supplemented with 1 % oil from the Recôncavo Baiano Basin, and the redox indicator 2,6-dichlorophenol indophenol (DCPIP) was added to evaluate their interaction in the presence of the oil as the sole carbon source. The DCPIP indicator was prepared as described by Hanson et al. (1993), and the degradation assessment was observed from the discolouration of the blue. This was used as a control in the experiment, and the isolated cultivation of each strain in the centre of the plate was prepared under the same conditions as the treatments.

The experiment was conducted with all possible combinations of two strains of fungi on a plate in random order with three replicates for each pair. A total of 21 combinations were assessed. The growth of the colony and the halo of discolouration of the culture medium were measured with the aid of a calliper, and visual macro changes were observed at intervals of 0, 3, 6, 9, 12 and 15 days.

The inhibition between fungi was estimated using the mathematical calculation of the inhibition index (Im), as a percentage of inhibition (%), according to the following formula:

$$Im = 100 - \left(\frac{MT}{MC}\right) \times 100$$

The variable MT corresponds to the arithmetic average of the test triplicate, and MC corresponds to the arithmetic average of the control triplicate, following the methodology of Menten et al. (1976). Thus, the antagonistic ability of each fungus was classified on an evaluation scale based on an index as follows: up to 20 % was classified as weak; > 20 % to ≤ 40 % was classified as moderate; > 40 % to ≤ 60 % was classified as positive; and > 60 % to ≤ 80 % was classified as strongly positive. The weak, moderate, positive and strongly positive classes were given scores of 1, 2, 3 and 4, respectively.

In addition, the classification of parameters was used according to an adaptation of the scale of Badalyan et al. (2002). The reactions classes were as follows: type A, mutual inhibition in mycelial contact; type B, inhibition at a distance; and type C, excessive growth. The following score was assigned to each type or subtype of reaction: A = 1; B = 2; C = 3; CA1 = 3.5; CB1 = 4;

CA2 = 4.5; CB2 = 5 and D = 0. The antagonism index (AI) was then calculated for each species of fungus using the following formula:

$$AI = A(n\,X1) + B(n\,X2) + C(n\,X3) + CA1(n\,X3.5) + CB1(n\,X4) + CA2(n\,X4.5) + CB2(n\,X5)$$

Where n = Frequency of each type or subtype of reaction

For the evaluation of species in the composition of the consortium, the percentage of resistance was also calculated as described by Morón-Ríos et al. (2017) as follows: % resistance = 100 %, which is the sum total of the inhibition of all species, – Σ ([growth of a given species of fungus in its respective control plate - growth of the species given to interact with another species] x 100/growth of a species on its respective control board).

The daily growth rate of each fungus was classified according to Asthana (1990) as follows: slow, 0–1 mm d -1 (3 points); moderate, 1–3 mm d -1 (2 points); or fast, 3-6 mm d -1 (1 point). The Kruskal-Wallis variance analysis and the Shapiro-Wilk normality test were Applied to the data. Data processing was performed using R and RStudio software version 3.4.2. The growth rate and the inhibition index were considered the response variables with three replicates.

8.4 RESULTS AND DISCUSSION

The qualitative and quantitative results of the 21 pairs of studied fungi are described in Table 11. “A” and “B” contain the information regarding the strains paired on the left and right, respectively.

Based on the Kruskal–Wallis variance analysis, no statistically significant difference was found (p value > 0.05) between the fungal growth rate and the control group. In other words, there was no difference in the studied microorganism growth with or without paired interactions during the 15-day incubation period at 30 °C. However, some differences were observed in terms of qualitative assessments and visual changes in 4 of the studied combinations. Most combinations did not exhibit visual inhibition as classified by Morón-Ríos et al. (2017) in the form of growth overlap, growth cessation before direct contact or mutual inhibition at distance, with the exception of pairings 7, 9, 10, 15 and 20 which were classified as antagonists (Table 11).

In paired culture, radial growth inhibition is attributed to substances released by one or both organisms, or to competition (TAPWAL et al., 2015). The R16 strain reacted antagonistically in combination 8 since it prevented N89 strain growth. In combinations 9 and 10,

the R16 strain released a dark-colored substance that was possibly a secondary metabolite resulting from the interaction. *Aspergillus* are mentioned in the literature as producers of a mycotoxin called ochratoxin A which can be produced in variable amounts by certain species of the group (NIELSEN et al., 2009). Ochratoxin A is a secondary metabolite that causes serious public health problems when it comes in contact with food due to its neurotoxic, immunotoxic, genotoxic and teratogenic properties (JUÁREZ-SEGOVIA et al., 2019). On the other hand, marine microorganisms cultivated in mixed fermentation can represent an effective source for inducing natural bioactive agents for the production of drugs, pesticides or biostimulants with unique structures and diverse bioactivities (LI et al., 2020; ANJUM et al., 2018). Strains of the *Aspergillus* genus are commonly found in the microbial community of soils and are responsible for the carbon cycle, being exploited biotechnologically as a source of enzymes (BAKER, 2006). Hu et al. (2011) observed greater production of laccase and β-xylosidase from the mixed cultivation of *Aspergillus niger* with *Phanerochaete chrysosporium*. Thus, even though the R16 strain exhibited antagonistic activity, it can be used for improved production of biotechnologically relevant enzymes and bioactives.

In combination 15, the fungus R26 produced spores that dominated the Petri dish. However, this is not reflected numerically through the inhibition index; the information of the radial growth from the inoculum positioned at the center of the Petri dish is adopted as an evaluation criterion, not adjacent formations. The same occurred in combination 9, where R16 grew on every plate, achieving greater growth when paired to control, leading to a different conclusion than that considering the pairing only in terms of Im (Figure 25- 1.2).

The growth of the A79 strain was reduced 72.35 % when paired with N89, even before direct mycelial confrontation, which constitutes inhibitory behavior. This finding corroborates the data found in Figure 25. *Byssochlamys* species have thermotolerant ascospores, have the ability to survive under conditions of low oxygenation and produce mycotoxins, such as patulin, bisotoxin A and bisoclamic acid (HOUBRAKEN et al., 2006). This may be related to an N89 strain that has characteristics of the genus *Byssochlamys.*

2,6-Dichlorophenolindophenol (DCPIP) is used in a colorimetric assay proposed in the literature because it exhibits a quick and simple response for evaluating the ability of microorganisms to degrade petroleum products from the oxidation of hydrocarbons and subsequent transfer of electrons to their respective acceptors (HABIB et al., 2017). In view of

this, the oxidation of DCPIP in the culture medium was used to assess the possible change in the functionality of each strain when sharing the source of the target nutrient, which in this case was oil. In all tests, discoloration of the redox indicator was observed; on Day 0, it was blue (Fig. 25A) and it gradually became colorless (Fig. 25B and 25C), except for the B side of pair 7. Even after the stability in fungal growth, the discoloration of the medium remained during the 15 days of incubation. This fact indicates that the microbial activity for oil degradation under these conditions can extend beyond this period. Antagonism in pairs 2 and 14 was expected to degrade the same fraction of oil; however, there was a reduction in the growth of both pairs without manifesting inhibitory reactions, demonstrating different selectivity. Several microorganisms have the ability to degrade complex mixtures of hydrocarbons through multiple metabolic pathways, and this range may be due to differences in gene diversity and protein structure (YANG et al., 2019). Most likely, the strains R11 and R16 have a set of genes that degrade using different metabolic pathways, and when judging the variation in the growth rate (0.5 cm) of R16, it appears to be more of a generalist or to have intermediate path genes.

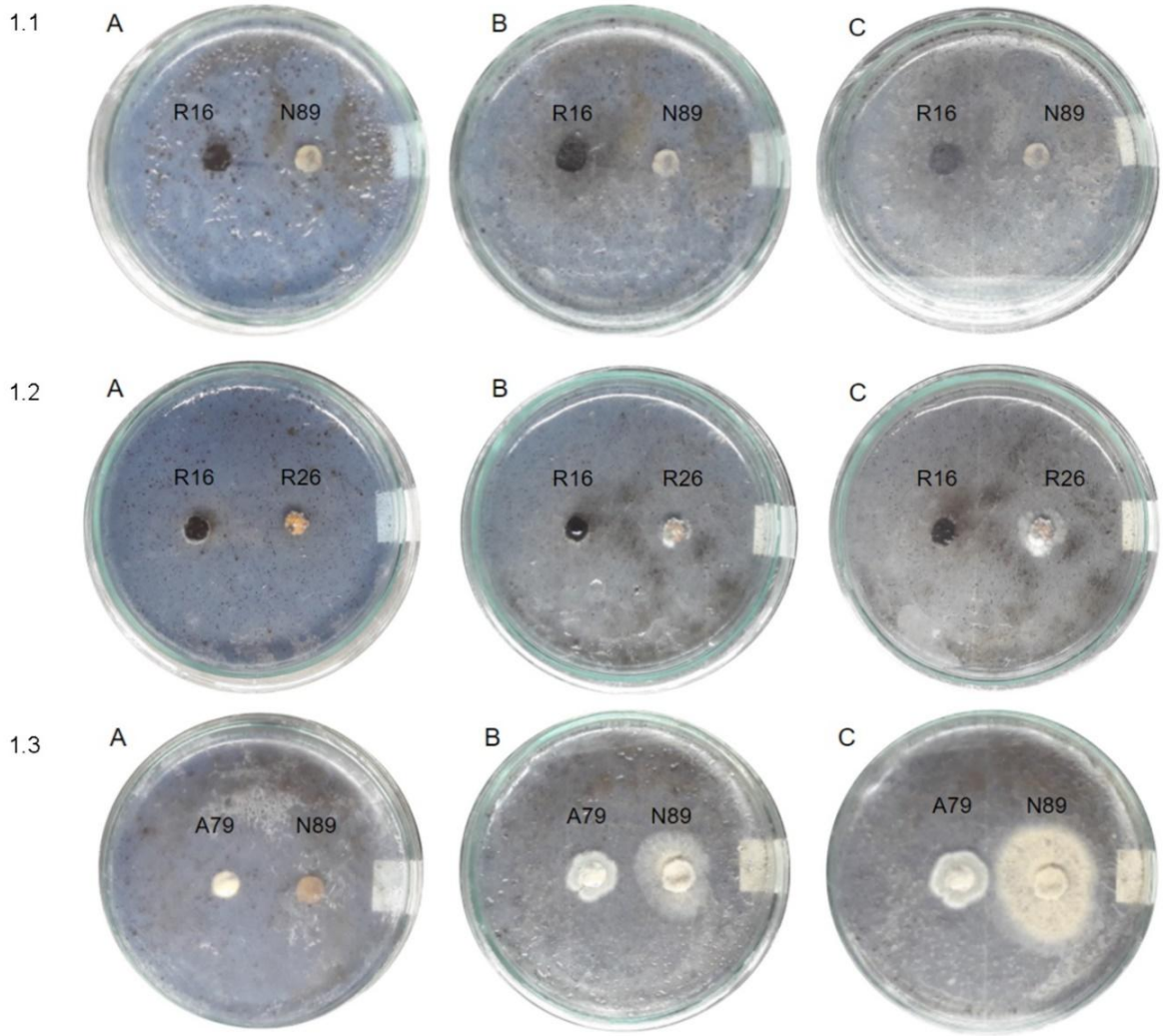
In Figure 26, the percentage of growth inhibition among the studied fungi is displayed, where the interaction was judged for the microorganisms in position A (left of the petri dish) and position B (right of the petri dish). The percentage of growth inhibition found for most pairings was less than 60 % in both positions, that is, only 14.3 % exhibited positive or strongly positive inhibition. Inhibitory rates up to 50 % in plating may be due to delimiting the space available for growth in relation to control and not necessarily due to survival mechanisms, such as the production of inhibitory metabolites or physical attack by microorganisms (BADALYAN, 2002). Thus, the antagonistic ability of each fungus in weak or moderate inhibitions does not preclude them from a consortium. Pairs 7 and 20 exhibited disharmonious relationships since the inhibition index was strongly positive (> 80 %) and positive (> 60 %), respectively. The total inhibition of the N89 strain by R16 in pair 7 indicates competition antagonism. The R16 strain is a fast-growing fungus that is able to degrade the lightest and heaviest fractions of oil; therefore, it is possible that the R16 strain consumed every available carbon source before the N89 strain could develop and prevented its growth. The A79 and N89 strains, when paired in plaque, exhibited antagonism during the two weeks of testing (Figure 25-1.3). The N89 strain suffered a reduction in development; however, it was classified as weak, with an observed value of 17.45 %.

Table 11 - Variables for determining in vitro antagonism by matching petroleum-degrading fungi

Strains		Oil fraction		Control Growth (cm)		Paired Growth (cm)		DCPIP oxidation		Interaction type
Nº	CÓDIGO	A	B	A	B	A	B	A	B	AB
1	R11XN89	5	3	3,00	5,33	2,23	4,00	+	+	D
2	R11XR16	5	5	3,00	3,67	1,70	3,17	+	+	D
3	R11XR26	5	4	3,00	3,17	1,90	3,90	+	+	D
4	R11XA79	5	2	3,00	2,17	2,13	3,75	+	+	D
5	R11XR31	5	3	3,00	1,87	2,10	1,87	+	+	D
6	R11XS40	5	1	3,00	2,50	2,30	2,90	+	+	D
7	R16XN89	5	3	3,67	5,33	2,83	0,00	+	-	CA2
8	R16XA79	5	2	3,67	2,17	2,30	1,57	+	+	D
9	R16XR26	5	4	3,67	3,17	1,33	2,33	+	+	CA1
10	R16XS40	5	1	3,67	2,50	2,67	2,30	+	+	B
11	R16XR31	5	3	3,67	1,87	2,00	1,75	+	+	D
12	R31XS40	3	1	1,87	2,50	1,67	1,77	+	+	D
13	R31XA79	3	2	1,87	2,17	1,93	1,97	+	+	D
14	R31XN89	3	3	1,87	5,33	1,00	2,87	+	+	D
15	R31XR26	3	4	1,87	3,17	1,60	3,75	+	+	CA2
16	S40XA79	1	2	2,50	2,17	2,17	1,73	+	+	D
17	S40XN89	1	3	2,50	5,33	1,50	3,00	+	+	D
18	S40XR26	1	4	2,50	3,17	2,00	3,77	+	+	D
19	A79XR26	2	4	2,17	3,17	1,13	3,53	+	+	D
20	A79XN89	2	3	2,17	5,33	0,60	4,40	+	+	B
21	N89XR26	3	4	5,33	3,17	4,25	3,50	+	+	D

Source: Author, 2022.

Figure 25 - Visual antagonistic interactions between oil-degrading fungi in dual Bushnell Haas cultures supplemented during the Day 0 (A), Day 7 (B) and Day 14 (C) incubation periods. 1.1 pairing number seven; 1.2 pairing number nine and 1.3 pairing number twenty



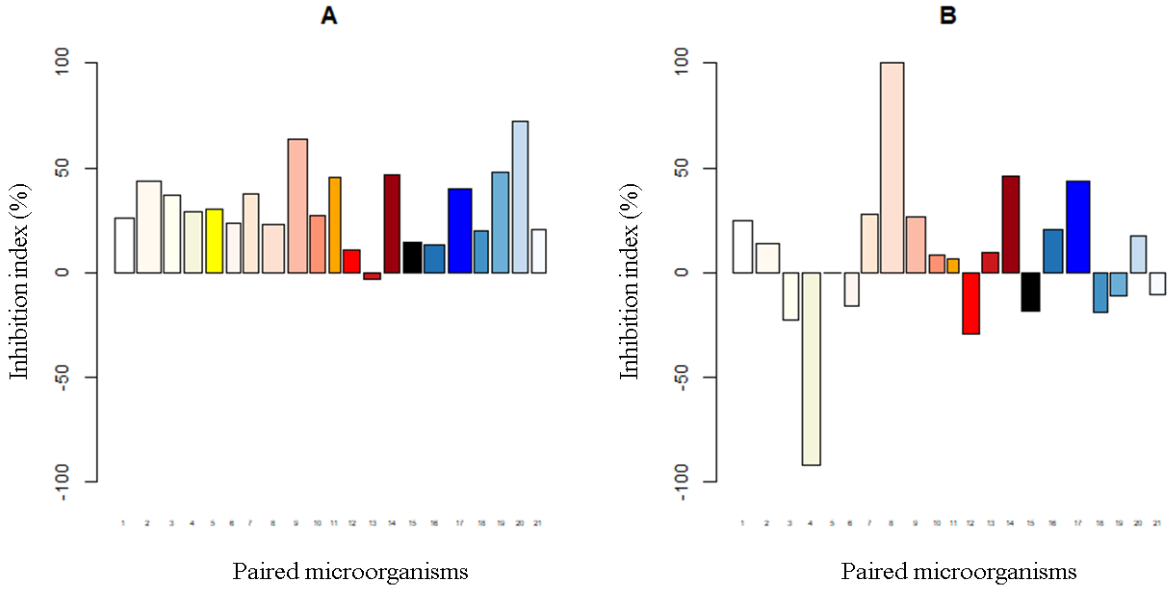
Source: Author, 2022.

Conversely, the A79 strain exhibited an inhibition rate of 72.35 %, which was classified as positive inhibition. Although there was no overlap of species, a confrontation zone was formed, preventing the growth of the A79 strain. Microbial antagonism is defined as the inhibition, deterioration or death of one microorganism species caused by the action of another, or a relationship between two populations in which one of them has a harmful or negative effect on the other. Meanwhile, synergism leads to protocooperation between species or two microbial populations, indicating that both benefit from the relationship. Thus, the R16 and N89 strains were classified as antagonistic based on the relationship observed. Pair 9 included the R16 and R26 strains; the inhibition rate for the R16 strain was 63.76 % and it had a greater development

(Figure 26), though the growth was dispersed and not reflected in the index, and there was evidence of a release of metabolites. It is possible that the R16 strain had a developmental advantage due to its capacity to degrade the three fractions of oil since the R26 strain consumes only the saturated fraction and the heteroatomic compounds. Thus, these fungi, when paired together, likely compete for the same carbon source necessary for growth, thus preventing their development (ABDALLAH et al., 2018).

On the other hand, pairs 3, 4, 6, 12, 15, 18, 19 and 21 showed no inhibition of the microorganisms in position B and, therefore, exhibited an expansion in their development (Figure 26). The A79 strain in pair 4 stood out with the highest negative inhibition index; it doubled in size when paired with the R11 strain. In this case, we assume that there was increased hydrocarbon mineralization since its growth increased. This phenomenon can occur when, because of their enzymatic and metabolic function, the microorganisms in the consortium are stimulated by the communities involved, which does not happen with a single strain (WANAPAISAN et al., 2018). Therefore, the cooperative interaction of different microorganisms can result in an increase in the bioavailability of the contaminant and consequently increase the rate of biodegradation.

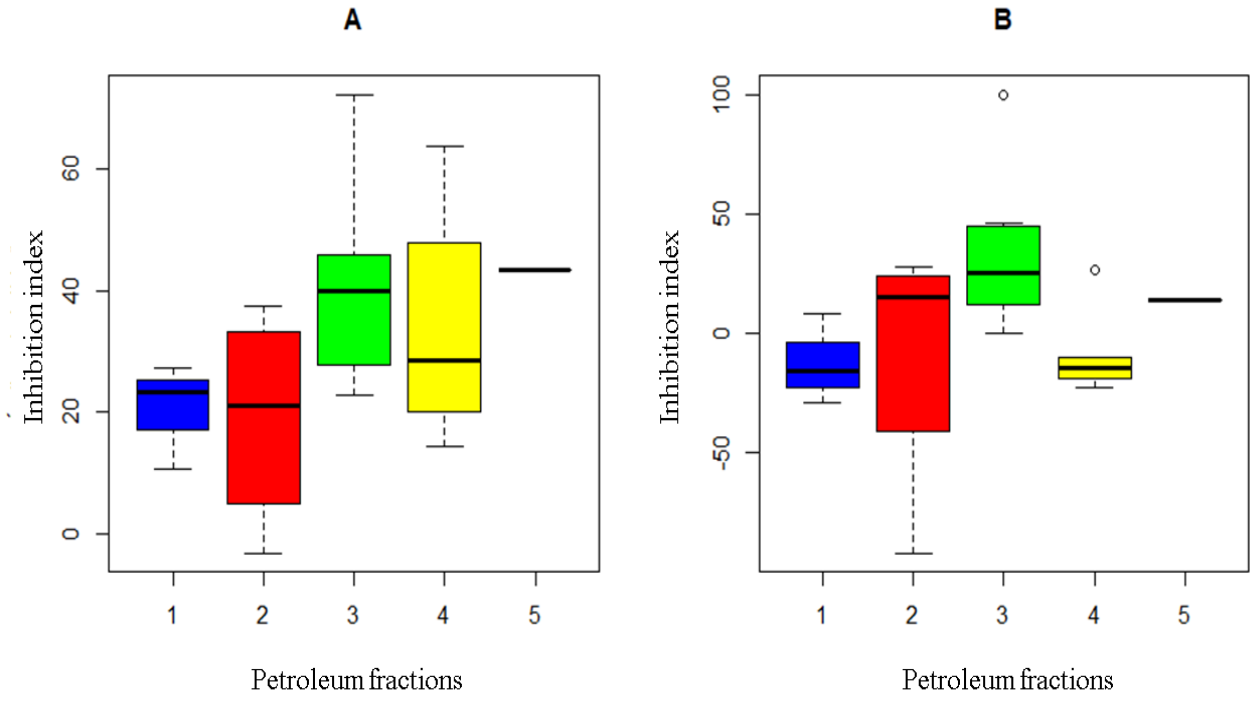
Figure 26 - Percentage of growth inhibition in the fungus versus fungus interaction. (A) Inhibition values of microorganisms in position A in relation to position B. (B) Inhibition values of microorganisms in position B in relation to position A



Source: Author, 2022.

In terms of oil degradation, saturated, aromatic and NSO substances are the most common and important hydrocarbon fractions in paraffin oil (AL-DAHHAN; MAHMOOD, 2019). Thus, when evaluating the conjuncture of the strains in Figure 27, it is noted that the classifications 2 (Aromatic), 3 (NSO compounds) and 4 (Saturated and NSO compounds) are those that present the greatest variation in the results of inhibitory percentage, contrary to those of rating 5 that showed lower. Among the degrading microorganisms of saturated fractions, aromatics and NSO compounds, less competition probably occurred because it is the ability to degrade all fractions of oil and therefore had greater availability of nutrients. The data showed that fraction 2, represented by strain A79, obtained a greater variation in data, with inhibition rates that ranged from negative (-92.87) to positive (72.35), demonstrating its distinct ability to adapt to different fungus-fungus interactions. Outliers detected in B for oil Groups 4, are represented by the only positive inhibition index that was found to be outside the statistical standard for the R26 strain.

Figure 27 - Variation in the growth inhibition index in relation to oil fractions. (A) Inhibition values of microorganisms in position A in relation to position B. (B) Inhibition values of microorganisms in position B in relation to position A



Source: Author, 2022.

The inhibition index cannot be used to infer the antagonism process in isolation since it is necessary to evaluate the macro- and microscopic changes of the microorganisms involved, as well as to test the joint functionality of the strain in question. Thus, the resistance test together with the classification of the interaction, growth information, and inhibition rate can provide support for the selection of microorganisms most suitable for forming a microbial consortium.

In this investigation, the 7 strains of filamentous fungi used exhibited various degrees of interaction with each other. The criterion for selecting the best strains was the lowest score achieved after classification of the parameters evaluated in Table 12.

The R26 strain achieved more impressive results when compared to the other strains with lower punctuation values in most of the studied parameters, confirming its importance as a bioproduct of hydrocarbon degradation. In addition, the combined scores for the rates of interaction, growth, inhibition and resistance, of the R31 and S40 were 104.93 and 130.06, respectively. Even so, the other strains also exhibited satisfactory results based on these parameters, so they can be considered promising agents for the formation of a microbial consortium.

In a consortium, the microorganisms are in direct contact and divide nutrients and, thus, they act in metabolic synergy; in contrast, in the context of oil degradation, a fraction is initially degraded by a certain species for subsequent degradation by another through cometabolism (AYDIN et al., 2017). Furthermore, mixing two or more strains can yield great advantages in the development of cleaning agents for polluted ecosystems. In the study of Chen et al. (2021), the *Penicillium citrinum* and *Citrobacter freundii* strains, in cocultivation, exhibited greater growth, and cell viability was maintained for up to five cycles, resulting in nitrate removal at levels above 80 %. This same synergistic behaviour was observed by Hechmi et al. (2016), who found increased production of *Byssochlamys nivea* in cultivation with *Scopulariopsis brumptii*, the subsequent removal of 95 % of pentachlorophenol and reduced toxicity in contaminated soil. Petroleum biodegradation increased from 75 % to 90 % by *Aspergillus fumitagus* when it was cultivated with *Aspergillus niger* (AL-JAWHARI, 2014). Thus, the cocultivation of the aforementioned strains in different concentrations and cultivation conditions should be encouraged to obtain a degrading agent with improved yield.

Table 12 - Evaluation of paired fungal reactions and corresponding values for each interaction parameter.

Strains	Growth	AI	Inhibition	Resistance	Total points
	2	0	13		229.73
R11				214.73	
R16	2	10	15	271.93	298.93
R31	2	4.5	8	90.43	104.93
R26	2	8	7	-65.90	-48.90
S40	2	2	9	117.06	130.06
	2	2	13		243.87
A79				226.87	
N89	2	6.5	15	282.51	306.01

Source: Author, 2022.

8.5 CONCLUSION

Although all the strains in this study have the ability to degrade petroleum, the R16, N89 and A79 strains exhibited antagonistic characteristics that can interfere with the degradation process and were, therefore, disqualified for use together. They can be further studied for their role in producing hydrocarbon degradation enzymes or mycotoxins for other purposes, such as for use in the food or pharmaceutical industry. They can also be used as biocontrol agents for the species tested. The R26, R31 and S40 strains, on the other hand, demonstrated strong potential for the assembly of a hydrocarbon-mineralizing consortium. The use of mixtures or alternations of these microorganisms is encouraged, along with an evaluation the degradation of undesirable organic compounds, to further improve the efficiency of the consortium.

9 BIOPROSPECTION OF LIGNINOLYTIC ENZYMES FROM MARINE ORIGIN FILAMENTOUS FUNGI

9.1 ABSTRACT

Fungi are excellent producers of extracellular enzymes. Therefore, the present study aimed to investigate the screening of marine fungi, which are laccase and manganese peroxidase potential producers, in solid fermentation for future applications in bioremediation processes of contaminated sites. For this purpose, two-level factorial planning was adopted, using time (6 and 15 days) and the absence or presence of oil (0 and 1 %) as factors. The semi-quantitative evaluation was carried out by calculating radial growth, enzyme activity and enzyme index by measuring phenol red or syringaldazine oxidation halo. The results showed that all the studied strains showed a positive result for manganese peroxidase production, with an enzymatic activity in solid medium less than 0.61, indicating a strongly positive activity. Through the enzyme index, the study also showed prominence for *Penicillium* sp. strains, with values > 2. The enzyme index increase in oil presence and the inexpressive use of the genera studied for ligninolytic enzymes production from crude oil demonstrated these data importance for fermentative processes optimization. Considering the ability of these strains to develop into recalcitrant compounds and the potential for manganese peroxidase production, they are indicated for exploitation in various bioremediation technologies, as well as other biotechnological applications.

Keywords: bioremediation, enzymatic activity, manganese peroxidase, mycoremediation

9.2 INTRODUCTION

The production of enzymes from microorganisms exposes great biotechnological, industrial and environmental interest due to its wide catalytic activities variety, large-scale production possibility, adaptation ease, renewability capacity and propensity for socioeconomic and environmental merits (BEHBUDI et al., 2021). Therefore, ligninolytic enzymes have a background for white biotechnology, with great potential for generating bio-products with high added value from natural biomass, following the green agenda (BILAL; IQBAL, 2020).

Ligninolytic enzymes (LEs) are made up of three main representatives: lignin peroxidase (LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13) and laccase (Lac, EC 1.10.3.2), along with others accessory enzymes (WONG, 2009) and have the function of catalyzing modifications or degrading lignin into less complex molecules (ASEMOLOYE et al., 2020). These enzymes have great versatility that covers a wide spectrum of use in bioremediation, and it is pointed out its complex recalcitrant compounds degradation and mineralization capacity, such as petroleum compounds and their derivatives (ASEMOLOYE et al., 2020), pesticides (ZENG et al., 2017), drugs, such as endocrine disrupters (ELDRIDGE et al., 2017) and polycyclic non-

steroidal anti-inflammatory drugs (BANKOLE et al., 2021), synthetic fragrances (VALLECILLOS et al., 2017), lignocellulosic residues (KUMAR; CHANDRA, 2020) textile effluent dyes (Rather et al. 2018) and also allows biocatalytic plastic recycling (WEI; ZIMMERMANN, 2017), bleaching paper (NATHAN et al., 2018) and biosensors production (SIDWABA et al., 2019) among others. Most residues cited above have in their composition compounds that can cause adverse effects to the natural environment and to humans, among them polycyclic aromatic hydrocarbons (PAHs), formed by two or more condensed aromatic rings and responsible for several biological effects, such as high toxicity, mutagenicity and carcinogenic potential (KADRI et al., 2017).

The bioremediation use as a strategy for affected areas recovery has advantages in being an ecologically correct and cheaper technique when compared to chemical and physical methods of remediation (KURNIATI et al., 2014). In recent years, interest in using fungi ligninolytic system as bioremediation agents has grown exponentially (RAO et al., 2014). This interest is mainly due to its oxidative efficiency, sustainable character, its low specificity to the substrate, feasibility of improving its production, stability and activity, and the possibility of immobilizing them when the use of microorganisms is limited (OGOLA et al., 2015).

Many *Aspergillus* sp. and *Penicillium* sp. fungi species are reported in literature as efficient in natural environments contaminated by organic pollutants bioremediation through extracellular production of LEs (LI et al., 2020). Marine environments are an extraordinary source of microorganisms diversity capable of adapting to extreme conditions, therefore their enzymes are very attractive in unusual bioprocesses (THEERACHAT et al., 2019). Thus, marine origin fungi have great potential for industrial application, once they are capable of producing enzymes with different physiological characteristics, such as tolerance to high rates of salinity and pH, high pressures, thermostability, psychrotolerance, thermostability and barophilicity (BONUGLI-SANTOS et al., 2015).

The demand for screening studies of new enzymes is a need that extends from today, in order to improve more competitive and sustainable production processes (ADRIO; DEMAIN, 2014). It is known that enzymes have different characteristics depending on the species, strains and cultivation conditions, therefore, although they have been extensively isolated and characterized from different natural sources, enzymes production system is a limitation for further large scale and reduced cost exploration (SHARMA et al., 2018). In this sense, it is

understood that for industrial processes development aiming enzymes production, initially the isolation and selection of microorganisms potentially producing enzymes with biotechnological interest is required, making bioprospecting studies fundamental (PALUDO et al., 2019).

The use of simple methods can be effective in microorganisms characterization and configure a quick and useful screening when evaluating individual fungi (HANKIN; ANAGNOSTAKIS, 1975). The direct correlation of radial growth diffusion and oxidation halo formed in solid culture medium can be used to determine the activity of extracellular enzymes (CESKA, 1971). Thus, the selection of undesirable compound degrading fungi through their enzymatic activity represents a viable evaluation strategy for obtaining promising biotechnology industry microorganisms. (LIN et al., 1991). Therefore, the present work aims to screen marine origin filamentous fungi that are laccase and manganese peroxidase potential producers in solid fermentation for future bioremediation processes applications in hydrocarbon contaminated sites.

9.3 MATERIALS AND METHODS

Five strains of filamentous fungi isolated from mangrove sediment in the Todos os Santos Bay, Brazil were obtained from the library of the Centro de Excelência em Geoquímica do Petróleo (LEPETRO) at the Universidade Federal da Bahia. Data derived from phylogenetic analyzes (supplementary file) identified these fungi as *Aspergillu*s sp. (R16), *Penicilium* sp. 1 (S40), *Penicilium* sp. 2 (R31), *Penicilium* sp. 1 (R26) and *Byssochlamy*s sp. (N89). These microorganisms were selected for their ability to tolerate / degrade petroleum hydrocarbons (LIMA et al. 2017, 2018) and therefore constitute a mixed microbial consortium protected by the patent with deposit number BR 10 2021 002341 4 (Lima et al. 2021), at the Instituto Nacional de Propriedade Industrial (INPI) and endorsed at the National Biotechnology Information Center (NCBI) under access numbers MW855899, MW855902, MW855901, MW855900, MW855903, respectively. They were grown on Sabouraud Dextrose Agar (SDA) (Kasvi®, Brazil) and maintained at 4 ° C for further analysis.

The production capacity of lignolytic enzymes was determined from the oxidation of specific compounds present in the culture medium (AGRAWAL et al., 2017), where the 0.1 % (m/v) syringaldazine solution was used for lacase (Lac) and the 2 mM $MnSO_4$ solution and 0.1 % (w/v) phenol red (Sigma-Aldrich®) for manganese peroxidase (MnP) according to guidelines adapted from the method described by Szklarz et al. (1989) and Kuwahara et al. (1984),

respectively. One disc (1 cm Ø) of pure colonies from each fungus was inoculated in the center of the Petri dish containing the Bushnell Haas medium (BH) (Difco®, United States) plus 1 % (v/v) of oil from the Recôncavo basin sterilized in UV for 15 min, for condition I and in the culture medium Sabouraud Dextrose Agar (SDA) (Kasvi®, Brazil) for condition II. Subsequently, the strains were incubated at 30 °C and changes were observed for 15 days.

The identification of positive enzymatic reactions occurred through the formation of a discoloration halo around the colonies. The semi-quantitative determination of the enzymatic potential was evaluated by measuring the diameter of the halo produced and the radial growth of the colony with a millimeter rule. The data were initially submitted to the calculation of the enzymatic index (IE), which reflects the ratio of the diameter of the halo to the diameter of the colony (HANKIN; ANAGNOSTAKIS, 1975) and subsequently the enzymatic activity (Pz) which corresponds to the ratio between the mean value the growth zone and the total growth zone plus oxidation of each strain (PRICE et al., 1982). Pz is classified and coded from the values obtained, where: it is considered negative when equal to 1, Pz = class 1; positive when <1> 0.64, Pz = class 2 and strongly positive when <0.64, Pz = class 3 (QUEIROZ; DE SOUZA, 2020).

The factorial design adopted was 2^2, with two replicates totaling 4 experiments for each strain. Two levels related to the lowest (-1) and highest (1) were adopted, where 6 and 15 days are related to time (factor 1) and 0 and 1 are for the absence of oil (factor 2). These experiments were carried out to predict better conditions for the fungi's ligninolytic enzyme index as a function of time and the presence or absence of oil in the culture medium.

The experiment was conducted with random sampling, with repetitions for each strain and cultivation condition. The data obtained were analyzed using the Minitab software version 20.2.0.0, where factor planning and normality and homogeneity tests of variance were applied, considering a value of $p \leq 0.05$.

9.4 RESULTS AND DISCUSSION

As shown in Figure 28, the strains when grown in BH medium corrected with manganese sulfate and phenol red became colorless, showing complete degradation of phenol red, in SDA medium the reaction was reversed from colorless to red in different shades. On the other hand, there was no change in culture media color which syringaldazine was used as a standard substrate (data not shown) for Lac. Therefore, the results obtained by observing the discoloration halo,

shown in Figure 28, demonstrated that *Aspergillus* sp., *Penicillium* sp. 1, *Penicillium* sp. 2, *Penicillium* sp. 1(R26). and *Byssochlamys* sp. has the capacity to produce manganese peroxidase in a solid culture medium, however it did not show activity for Lacase. The same result was found by Vipotnik et al. (2021), where it was not possible to detect Lac for *Penicillium chrysogenum* in 2 weeks of incubation in solid fermentation under the influence of pH 7.

Lignolytic enzymes production is influenced by several factors, such as carbon and nitrogen source, growth conditions and the effect of aromatic compounds (SCHNEIDER et al., 2018), as well as genetic, physiological or ecological specificities of each fungus (ELISASHVILI et al., 2010). It is known that catalytic activity requires the presence of cofactors, such as metals and coenzymes, as well as an ideal pH range, so their absence can affect the production and activity of enzymes (COELHO et al., 2008). In this sense, it can be inferred that, probably, the cultivation conditions were not satisfactory for laccase production, since the ideal pH for production in *Penicillium cyclopium* and *Penicillium digitatum* was considered acid close to 5 and temperature of 25 °C (EL-SHORA et al., 2008), different from what was carried out in this study, pH 7 and 6 ± 0.2 at 30 °C for BH and SDA, respectively. On the other hand, the results found in Kumar et al. (2016) are divergent, since *Pinctada martensii* obtained optimum laccase production conditions at 35 °C in a pH 7, indicating optimum conditions variation for each strain. As for the genus *Aspergillus*, *Aspergillus. flavus* Lac production was found from in solid medium, both at acidic pH and at pH close to neutrality (pH from 3.5 to 7.5) and in temperature variations of 20 °C at 35 °C (AFTAB et al., 2018). Notwithstanding were the results of Hu et al. (2011), where he obtained enzymatic activity for Lac in *Aspergillus niger* and *Aspergillus oryzae*, both in individual culture and in co-cultivation at 25° C or 30° C in a pH 6.0. One of the major differences between the works mentioned and the current one is in substrate, for this reason we can infer that, although it was possible to observe the production of Lac by other authors at pH 7, the optimal pH also depends on the substrate used (FUKUSHIMA; KIRK, 1995). In this sense, once most fungi laccases are preferably produced at a pH close to 3 using 2,2'-Azino-Bis (ABTS) as a substrate (KUMAR et al., 2016), it is believed that syringaldazine can also be similar and therefore resulted in the non-detection of Lac in the conditions provided. Furthermore, pH in acidic conditions can favor the development of filamentous fungi and assist hydrocarbons degradation (MACIEL et al., 2010). Regarding *Byssochlamys* genus, although it is widely used in bioremediation strategies (MANN et al., 2010, HECHMI et al., 2016) some species, such as

Byssochlamys nivea are not able to produce the enzyme laccase or lignin peroxidases (BOSSO et al., 2015), which corroborates our findings.

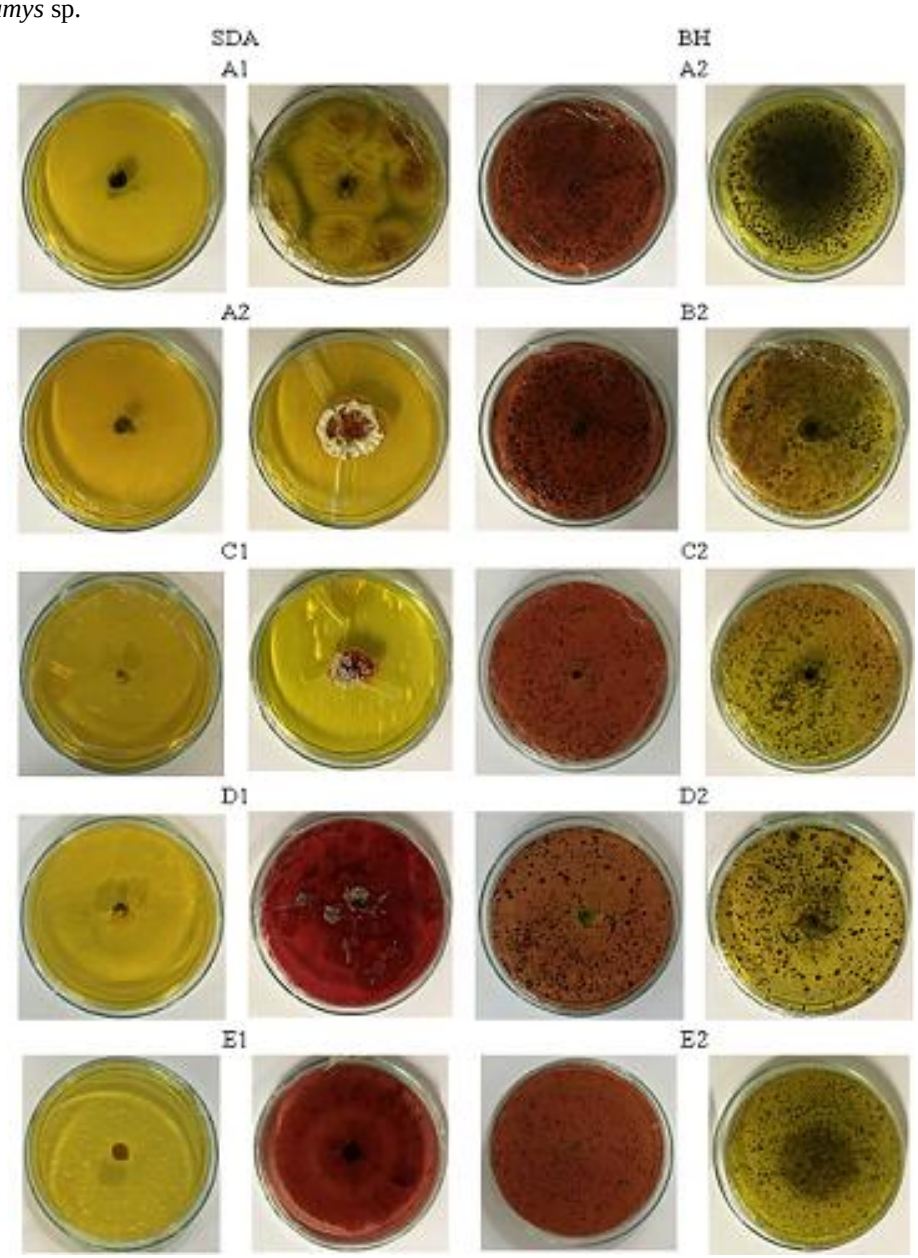
Laccase has a limited effect on bioremediation due to its specificity forlignin phenolic units and thus some substrates cannot be directly oxidized by Lac (THEERACHAT et al., 2019), in these cases the limitation can be overcome with the addition of mediators, as well as some other enzymes, such as: feruloyl esterase, lipases, aryl-alcohol oxidase, catechol 2, 3-dioxigenase, quinone reductases (KUMAR; CHANDRA, 2020). Thus, it is necessary to optimize the adaptable subtract condition to these fungi, as well as to test different redox mediators concentrations, such as 2,20-azino-bis and p-cumárico acid, 1-hydroxybenzotriazol (BANKOLE et al., 2021) or the inclusion of inductors, such as copper sulfate (DE SALAS et al., 2019, FERRARONI et al., 2017), ethanol, methanol, isopropanol, acetone, tween 80, glycerin, glucose, triton X, sulfate iron, calcium chloride, sodium nitrate (AFTAB et al., 2018), in order to improve the target enzyme activity.

Through Figure 28 it is possible to observe the culture media color change around the strains colony diameter after 15 days of incubation. manganese peroxidase is a manganese-dependent enzyme, which, from the manganese oxidation enables the subsequent oxidation of phenolic compounds (GLENN et al., 1986). Thus, this enzyme synthesis is indicated through the phenol red oxidation and subsequent color change, as shown in Figure 28. The results showed the enzyme presence in all fungi in a culture medium with 1 % oil. This enzyme production in response to crude oil increase in *Aspergillus niger* corroborates the data found in Asemoloye et al. (2018), which acquired 70 U.mL $^{-1}$ of MnP when produced in 5 % of oil. Similar results were observed in Maciel et al. (2010), using diesel oil as a substrate and reached a production of 60 U.L and 7 U.L for MnP from *Penicillium* sp. and *Aspergillus tamarii*, respectively. For *Byssochlamys* genus, 0.17 IU.mL of MnP production was also observed, according to M'barek et al. (2019).

Among fungal peroxidases, MnP has great ecological importance, mainly due its plant biomass conversion and recalcitrant compounds degradation (KELLNER et al., 2014). This enzyme is extensively explored biotechnologically because it mediates textile dyes biocatalysis reactions (ZHANG et al., 2020), in addition to presenting high petroleum hydrocarbons degradation rates (BECARELLI et al., 2019). Therefore, the cited fungi demonstrated ability to secrete MnP, which gives them great potential for environmental and industrial applications, such

as pulp and paper industries for bio-bleaching processes, pulp production and effluent purification (SALDARRIAGA-HERNÁNDEZ et al., 2020) or in the food industry, producing natural aromatic flavors (LI et al., 2015). Furthermore, these enzymes can play a vital role in biofuels production and other biochemicals, since they oxidatively depolymerize lignin and leave the polysaccharides intact (MACIEL; RIBEIRO, 2010).

Figure 28 - Phenol red discoloration halo seen from back by fungal strains in SDA medium (1) and BH medium (2) in 1 day and after 15 days of incubation. a) *Aspergillus* sp. b) *Penicillium* sp. 1 c) *Penicillium* sp. 2 d) *Penicillium* sp. 1(R26) e) B*yssochlamys* sp.



Source: Dantas, 2022.

Table 13 describes the enzyme index and the enzyme activity found for manganese peroxidase of studied fungi at the end of 15 days of incubation. The enzymatic activity of MnP varied between 0.24 and 0.61, grouping all strains as class 3. Since the Pz is less than 0.64, the strains are classified with strongly positive activity, which is, the lower the value of Pz, the greater the potential for production of the enzyme and therefore should be considered excellent producers of the enzyme in question.

Since *Aspergillus* sp. showed enzymatic activity of 0.61 and 0.37 in SDA and BH media, respectively, it is believed that it provides favorable characteristics for MnP production and, consequently, bioproducts production. No reports were found in the literature regarding Pz, however it is known that *Aspergillus* sp. are well known for secreting large amounts of enzymes (HERNÁNDEZ-MARTÍNEZ et al., 2011) and for this reason they are often mentioned in several studies as efficient producers of lignolytic complex enzymes, among them the work of Reis et al. (2019) where *Aspergillus niger* is cited as a viable alternative production of multiple enzymes, including MnP and Lac. In addition, the *Aspergillus niger* strain can be adopted as a biodegradable agent for complex compounds, such as effluent discoloration composed of reactive yellow and reactive red dye, reaching 98.62 % and 92.42 % removal, respectively, as described by Salem et al. (2019), and as an alternative, *Aspergillus niger* it can also biodegrade the Congo Red dye, reaching a discoloration rate of 97 % as a result ligninolytic enzymes action, where MnP plays a crucial role in discoloration (ASSES et al., 2018).

All three *Penicillium* strains showed favorable results to production MnP, with increasing values of 0.31, 0.50, 0.53 on SDA media and 0.24, 0.25, 0.35 on BH media. Although the strains potential mentioned, in terms of volume, is not known, many *Penicillium* fungi have a great consolidated biotechnological role to degrade different compounds, as well as in Govarthanan et al. (2017) work, where he records in his study with *Penicillium* sp. obtaining 1.0492 U of crude MnP enzyme in the degradation process of decane, butylbenzene, dodecane, naphthalene, acenaphtene, octane, ethylbenzene and benzo [a] pyrene. In contrast, Chen et al. (2019) obtained a production range of 4.68–23.31 $U.mL^{-1}$ of MnP from *Penicillium simplicissimum* on triphenylmethane discoloration. Thus, this statement adds relevance to the isolates studied for the degradation of undesirable aromatic compounds.

Although there are few reports in the literature regarding the production of ligninolytic enzymes by *Byssochlamys* strains, the genus is mentioned as adapted for development in

environments contaminated by hydrocarbons (RADWAN et at., 2018) and is often pointed out as responsible for corrosion of metals (BLANEY, 2007). However, *Byssochlamys nivea* is mentioned as efficient in mycoremediation process of sites contaminated by aromatic compounds, such as pentachlorophenol (HECHMI et al., 2016) and metabolizing biodiesel and petroleum diesel (YE et al., 2017). Representatives of this genus also have genes involved in aromatic compounds and n-alkanes degradation, including cytochrome P450 alkane hydroxylase, cytochrome P450 monooxygenase, aromatic ring-opening dioxygenase, salicylate hydroxylase, 2-haloacidalogenase, benzyl alcohol dehydrogenase, benzoate 4- monooxygenase and dimethylsulfide monooxygenase (RADWAN et al., 2018). White rot fungi ability to degrade aromatic compounds is attributed to MnP and Lac activity, which is often focused on Basidiomycota, among them *Aspergillus* and *Penicillium*, however Ascomycota representatives such as *Byssochlamys nivea* are neglected (HECHMI et al., 2016). Therefore, the data from this study may be a stimulus for further investigations regarding these enzymes production from *Byssochlamys* genus and the strain exploration for bio products productions for white biotechnology.

Enzyme activity index is one of the most used semi-quantitative parameters to evaluate the production of enzymes by microorganisms in a solid medium (REZENDE et al., 2013). Based on this assumption, all the studied fungi can be considered manganese peroxidase producers, according to the data shown in Table 13. However, *Aspergillus* sp. and *Byssochlamys* sp. strains showed activity below 2 in SDA and BH medium, on the other hand, *Penicillium* sp.1 (R26) showed an enzyme index greater than 2 in both media as shown in Table 13. It is believed that microorganisms can be considered potentially viable for biotechnological use when they have an enzyme index > 2.0 (STAMFORD et al., 1998), generating relevance for *Penicilium* sp.1 (R26). It is assumed that the higher the enzyme index, the greater the enzyme production and, consequently, the greater degradation of target compounds. In this sense, the information found may be in agreement with the data presented by Abdullah et al. (2020), which describes indigenous fungi dominance on soil contaminated by crude oil belonging to *Penicillium* and *Aspergillus* genus and indicating *Penicillium* sp. as more efficient in breaking polycyclic aromatic hydrocarbons, reaching levels between 25 % to 32 % compared to 13 % to 19 % when mineralized by *Aspergillus* sp.

When observing the colony diameter values (Table 13), *Aspergillus* sp. and *Byssochlamys* sp. showed greater growth, reaching 9.0 and 4.8, 7.8 and 5.9, respectively. Thus, it is noted that colonies with a higher enzyme index were not necessarily the same strains that showed greater radial growth, that is, the microorganism development is not directly proportional to the target enzyme production.

Table 13 - Average and standard deviation of the enzyme index and enzymatic activity of strains for MnP in solid medium in 15 days of incubation.

	Enzymatic Index (IE)		**Enzymatic Activity (Pz)**		**Radial diffusion (cm)**	
Microorganism	**SDA**	**BH**	**SDA**	**BH**	**SDA**	**BH**
Aspergillus sp. 1	0.64 ± 0.03	1.64 ± 0.21	0.61 ± 0.01	0.37 ± 0.03	9.00 ± 0.00	4.85 ± 0.21
Penicilium sp. 1	0.97 ± 0.03	1.85 ± 0.27	0.50 ± 0.03	0.35 ± 0.00	3.50 ± 0.28	2.00 ± 0.00
Penicilium sp. 2	0.88 ± 0.17	3.15 ± 1.32	0.53 ± 0.05	0.25 ± 0.08	2.55 ± 0.35	2.50 ± 0.42
Penicilium sp.1(R26)	2.21 ± 0.22	3.18 ± 0.39	0.31 ± 0.02	0.24 ± 0.02	4.10 ± 0.42	3.00 ± 0.14
Byssochlamys sp.	1.15 ± 0.02	1.53 ± 0.14	0.46 ± 0.00	0.42 ± 0.02	7.80 ± 0.14	5.90 ± 0.56

Source: Dantas, 2022.

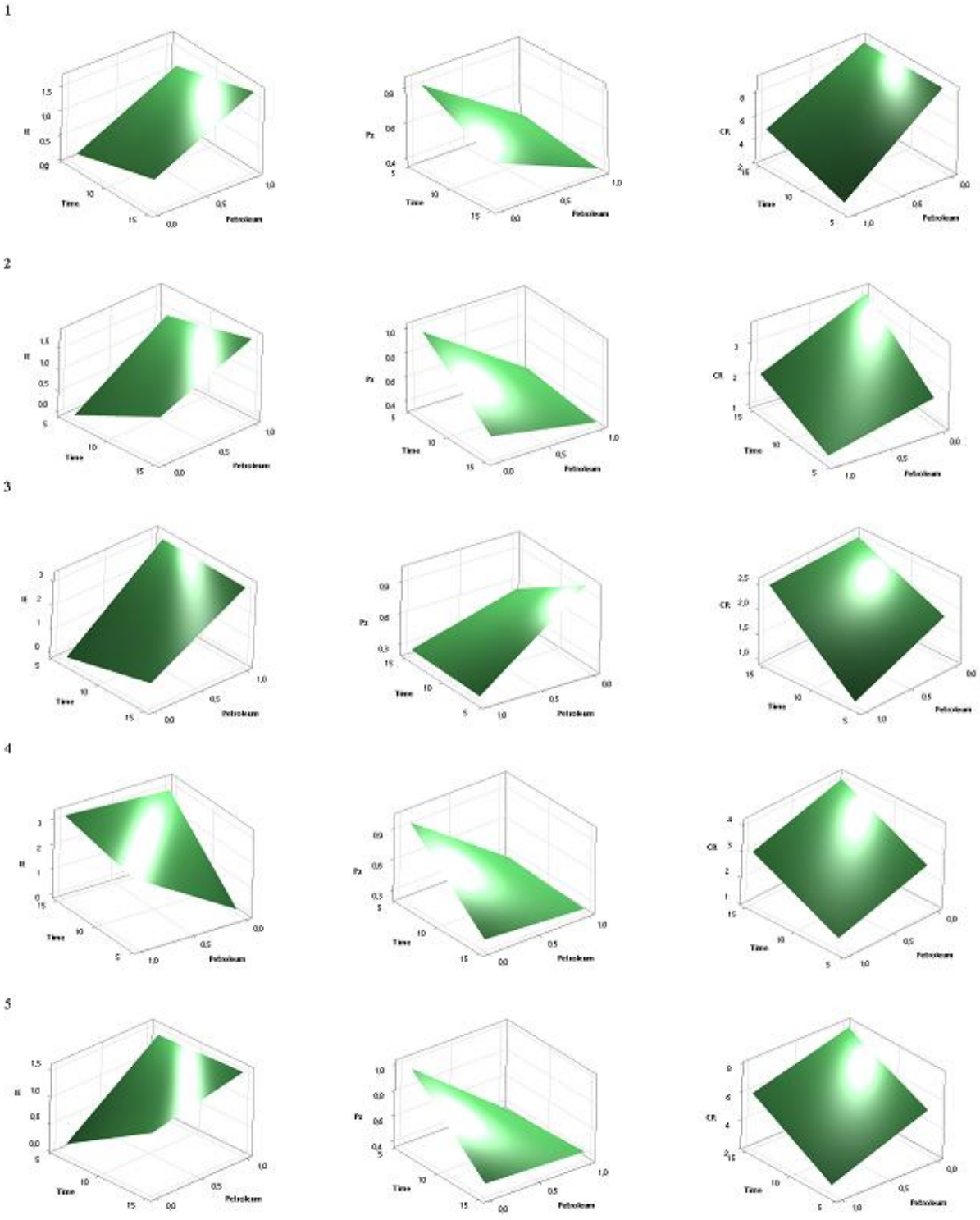
As shown in Figure 29, we can see the experimental design results for all the five strains studied. Time and absence or presence of oil in relation to IE significance were the main effects factors, with factor 2 being more significant, however the interaction factor between them was not statistically significant. Those factors' effect was positive, that is, the longer the time and the presence of oil, the better IE results in *Aspergillus* sp. and *Penicillium* sp. 1 (Figura. 29.a and 29.b). As for *Penicillium* sp. 2, only the oil presence showed significance (p value = 0.004), with a positive effect of 2.73 yield. On the other hand, in *Penicillium* sp. 1 (R26) and *Byssochlamys* sp., all factors and interaction positively influenced the enzyme index. In this sense, it is worth increasing oil concentration to obtain better EI results. Similar results were found by Chen et al. (2019), where enzymatic assays revealed that the activity of manganese peroxidase in *Penicillium simplicissimum*, was significantly induced in cultures supplemented with the contaminant when compared to the control (without contaminant). There are few reports in the literature regarding lignolytic enzymes production from petroleum products or even from crude oil in solid media. However, solid state fermentation (SSF) has been considered as a promising alternative for obtaining enzymes, as it offers a simpler fermentation medium, can provide higher productivity, allows greater control of microbial contamination, uses less water and consequently produces less

waste liquid (ATAGANA et al., 2006). In addition, SSF has great potential for capital reduction in further processing and production, especially when filamentous fungi and industrial waste are used and, therefore, assists in the reuse and recovery of this waste, mitigating environmental impacts arising from improper disposal or accidents (PIRES et al., 2020). Therefore, enzyme production in solid fermentation, with desirable characteristics for bioremediation, would help to develop bioprocess technology to obtain bioproducts useful in reducing pollutants toxicity.

For Pz, all effects were statistically significant, including factor 1 interaction with factor 2. However, the effect was negative, as expected, suggesting that the longer the time and the presence of oil, the lower the Pz value in *Aspergillus* sp., *Penicillium* sp. 1, *Penicillium* sp. 2, *Penicillium* sp. 1 (R26), and *Byssochlamys* sp., consequently the enzyme activity will be inclined towards strongly positive. The Pz data corroborate the IE findings, showing an inversely proportional relationship. In this sense, the increase in IE and the reduction in Pz demonstrate better results for the evaluation of the studied strains. The Pz and IE indexes were not previously used to evaluate the production of MnP in solid culture medium, however it represents a simple and efficient strategy to indicate studied species potential. Therefore, this method makes it possible to increase the range of screening options for fungi that produce ligninolytic enzymes for laboratories which does not have robust equipment.

Penicillium sp. 2 growth showed significance ($p = 0.005$) for factor 1, that is, it was influenced only by time with a positive effect. Apparently, both media did not provide a significant difference for the strain development. There was no change in growth, but there was an increase in IE when in BH medium. Factors effects and their combination for radial growth (CR) in *Aspergillus* sp. showed a p-value lower than 0.05, and therefore, demonstrated significance in this strain development. On the other hand, the effect was negative in oil presence, that is, the strain grew more in the SDA culture medium than in BH medium, but IE values were favored. The same occurred for *Penicillium* sp. 1., *Penicillium* sp. 1 (R26) and *Byssochlamys* sp., however the factors interaction was not significant. Thus, even though these strains' growth has declined, the contaminant interference allowed to obtain better EI and Pz values, emphasizing the evaluating importance of agreement between the variables studied. This result was also verified by Heinz et al. (2015), which highlights the need to evaluate the strain as a good enzyme producer, taking into consideration both microorganism growth in culture medium, as well as the enzyme index data.

Figura 29 - Three-dimensional response surface plot for MnP production using *Aspergillus* sp. (a), *Penicillium* sp. 1 (b), *Penicillium* sp. 2 (c), *Penicillium* sp. 1R26 (d) and *Byssochlamys* sp. (e) in solid fermentation



Source: Dantas, 2022.

A possible alternative to improve production in less time would be to increase the tested fungal biomass or change the contaminant concentration, evaluating degradation. Reports on fungi production in co-culture obtaining favored biomass over monoculture and the interaction between species reflects a greater tolerance to contaminant inhibitory effects (HECHMI et al., 2016), leading us to believe that these enzymes production limitations can be overcome by fermentation with multiple colonies. Such characteristics can be observed by other authors, such as Benoit-Gelber et al. (2017) where co-cultivation of *Aspergillus niger* with *Aspergillus. oryzae* resulted in a more complex enzyme profile, Hu et al. (2011) obtained higher enzyme production from mixed cultivation of *Aspergillus niger* with *Phanerochaete chrysosporium*, Zhao et al. (2019) reported the co-cultivation of *Penicillium oxalicum* and *Trichoderma reesei* under solid state fermentation to produce enzymes of biotechnological interest and obtained yields up to 4 times higher and Hechmi et al. (2016) obtained increased production with *Byssochlamys nivea* in cultivation with *Scopulariopsis brumptii*. In addition, microorganisms production in mixed cultures becomes a promising alternative for identifying new enzymes or compounds (WÖSTEN, 2019). Moreover, manganese peroxidase multiple production by the five strains can be encouraged to maximize the yield of the process.

9.5 CONCLUSION

Through the results obtained it was possible to infer that the studied fungi exhibited ligninolytic enzymatic activity, showing a capacity for the production of the enzyme manganese peroxidase with strongly positive enzyme activity values, with values of IE> 2 for the genus *Penicilium*, thus providing a scope for their future application for the purpose of bioremediation of environments impacted by oil and / or its derivatives. The factorial design indicated that the enzyme index was increased in solid state fermentation when in BH medium plus oil, indicating the potential of this contaminant in the production of the MnP enzyme. Taking into account that fungi of marine origin can be an important resource for use in biotechnological processes and knowing the abilities of the strains studied to develop in the presence of petroleum hydrocarbons, the results of this study stimulate further investigations on the production of ligninolytic and other enzymes, in addition to concentrating investigations on the characterization of these enzymes and on the optimization of the high biomass production process, in order to develop a bioproduct with multiple degradation functions.

10 PREVISION FUNCTIONAL OF PROTEOMIC DIVERSITY OF MANGROVE MICROBIAL CONSORTIUM

10.1 ABSTRACT

Mangrove microbial communities play several important roles in ecosystem maintenance and represent an important source of bioproducts for biotechnological applications in various industrial processes. In this work, an in silico proteogenomics approach was applied in an autochthonous mangrove microbial consortium, aiming to identify specific enzymes and functions for bioremediation. The study was carried out through the Universal Protein Resource and National Center for Biotechnology Information database, using identification sequences as a reference from 24 fungi of *Aspergillus*, *Allophoma*, *Byssochlamys*, and *Penicillium* genera and 9 bacteria of *Bacillus*, *Brevibacillus*, *Stenotrophomonas*, and *Pseudomonas* genera to investigate organic compound degrading enzymes, such as laccase, manganese peroxidase, lignin peroxidase, alkane hydroxylase, catechol 1,2 dioxygenase, catechol 2,3 dioxygenase, and protocatechol 3,4 dioxygenase. Several putative enzymes involved in aromatic compound degradation were found, except for manganese peroxidase and lignin peroxidase. Among the studied strains, only *Allophoma* sp.1 did not show results in the investigated database, demonstrating that its mechanisms may be poorly studied; on the other hand, *Bacillus* sp. were the most representative. The results showed a high metabolic exchange between fungi and bacteria related to laccases, catechol 1,2 and protocatechol 3,4 dioxygenase. Phylogenetic analysis indicated clade branch formations based on the intradiol or oestradiol ring cleavage action mechanism. Although the specific functions are merely suggestive, the relevance of the consortium species is proven in the literature regarding the presence of multiple enzymatic systems useful for bioremediation, and they have been shown to be favourable candidates for biotechnological process of petroleum biodegradation and other recalcitrant compounds.

Keywords: Enzymatic biodegradation, dioxygenases, microbial enzymes, laccase.

10.2 INTRODUCTION

Mangroves are transitional coastal ecosystems characteristic of tropical and subtropical regions. Brazil is one of the most abundant countries in mangroves in the world and is home to promising regions for microorganism prospectiong studies (GRATIVOL et al., 2017). The microbial community found in mangroves plays vital roles in maintaining the ecosystem and its productivity (AZIZ et al., 2017), such as nitrogen fixation, methanogenesis, and metabolization of organic compounds (MAO et al., 2015). To adapt to varying conditions of salinity and temperature, these microorganisms produce compounds with unique biological properties, providing them with advantageous characteristics for application in industrial processes (KATHIRESAN, 2019).

The mangrove microbiota represents an important source of bioproducts as they have a high potential enzymatic arsenal for use in new bioactive metabolite and biocatalyst research (CHANTARASIRI et al., 2021). Fungi as a group comprises species capable of producing and secreting enzymes useful for bioemulsifier, organic acid, and antibiotic production (VIGNESHWARAN et al., 2016). In addition to the fungal group, bacteria represent an important source of natural substances, due to their great genetic and metabolic variety (VIGNESHWARAN et al., 2016). Mangrove sediment is composed of a wide variety of microorganisms, including the order *Actinomycetos*, which produces primary and secondary metabolites that are valuable for industry (OMRANI, 2018). The structural and functional diversity of bacteria allows a diversity of bioactive metabolite production, such as highly applicable biosurfactants and bioemulsifiers (ARAÚJO et al., 2020). These microorganisms gathered in the form of microbial consortia can provide catabolic actions that are presumed to cooperate synergistically with undesirable degraded organic compounds, such as industrial dyes, waste from the paper industry waste, petroleum, and derivatives (HASSANSHAHIAN et al., 2020). Thus, they are considered crucial for efficient biotechnological tool composition both in terms of biostimulation and bioaugmentation (HASSANSHAHIAN et al., 2020).

The degradative action of microorganisms to transform recalcitrant organic compounds is related to oxidoreductase enzymes, such as lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac), and can occur through a single enzyme or a fungi and bacteria enzymatic cocktail (FANG et al., 2018). They play a crucial role in bioremediation, as they act by depolymerizing the target compound, through the ability to transform complex organic molecules into simpler compounds and use them as a source of carbon and energy (FANG et al., 2018). These enzymes are often found mainly in fungi of *Aspergillus* and *Penicillium* genera (GONZALO et al., 2016). Bacterial strains also revealed metabolic capabilities to biotransform organic compounds, including *Pseudomonas*, *Bacillus*, *Stenotrophomonas*, *Rhodococcus*, and others (BRZESZCZ; KASZYCKI, 2018). During their metabolism, these microorganisms can produce extracellular enzymes that have lipolytic activity and act at the water-oil interface (CHAKRABORTY et al., 2015). However, microorganisms capable of mineralizing the same compound can act under different mechanisms differently as a result of expressed enzyme composition, and when in harmony, they can act with high complementarity through metabolite exchange and acquisition (PUENTES-TÉLLEZ; SALLES, 2018).

Microbial communities associated with the bioremediation of degraded areas may differ in taxonomic classification, but little is known about their respective functional activities. Microbial proteogenomic bioprospecting has emerged as a conciliatory strategy with the potential to generate great economic impact in the pharmaceutical, biofuel, and fine chemical industries (MOUAFI, 2016). These analyses provided starting points for evaluating functional diversity in similar sequences from protein families and species-specific sequences within a similar group of microorganisms (KUMAR et al., 2018b). Understanding the relationship between the diversity and function of each strain allows the design of a configuration of functionally diverse microbial consortia to effectively carry out a variety of processes (PUENTES-TÉLLEZ; SALLES, 2018).

Therefore, the characterization of the individual enzymatic functions of strains is a useful tool not only for the biocatalysis of petroleum but also for other xenobiotic compounds. In this sense, this work aimed to carry out an in silico evaluation of organic compound degrading enzymes in an autochthonous mangrove microbial consortium of biotechnological interest to indentify these enzymes specific functions in the environmental bioremediation process.

10.3 MATERIALS AND METHODS

Samples of thirty-three autochthonous microorganisms from mangroves located in Todos os Santos Bay northern portion, in São Francisco do Conde, in the state of Bahia, Brazil, with organic compounds degrade ability, more specifically petroleum hydrocarbons and were selected for proteogenomic study *in silico* based on information found in Lima et al. (2018). The studied strains constitute a microbial consortium protected by patent with deposit number BR 10 2021 002341 4 (LIMA et al., 2021), at the Instituto Nacional da Propriedade Industrial (INPI). The strains nucleotide sequences in question were granted by Centro de Excelência em Geoquímica do Petróleo (LEPETRO), located at Instituto de Geociências (IGEO) of Universidade Federal da Bahia (UFBA), identified by molecular tools and endorsed under secrecy at the National Center of Biotechnology Information (NCBI) as described in Menezes-Neto et al. (unpublished data). Among the strains there are 24 fungi named under the codes and their respective accession number R2 - (MW865711), R11 - (MW855898), R16 - (MW855899), R26 - (MW855900), R27 - (MW865712), R28 - (MW865713), R30 - (MW865714), R33 - (MW865715), R31 - (MW855901), S36 - (MW865716), S38 - (MW865717), S39 - (MW865718), S40 - (MW855902), S41 - (MW865719), S45 - (MW865720), S52 - (MW865721), A79 -

(MW865722), A80 - (MW865723), A83 - (MW865724), A84 - (MW865725), N82 - (MW865726), N89 - (MW855903), N96 - (MW865727), N101 - (MW865728) and N102 - (MW865729), and 9 bacteria PD6 - (MW881201), RB4 - (MW881197), RC6 - (MW881199), PD8 - (MW881203), RA2 - (MW881196), RC5 - (MW881198), PD7 - (MW881202), PD5 - (MW881200) and OH4 - (MW881204).

All taxa were preliminarily screened for laccase (Lac), lignin peroxidase (LiP), manganese peroxidase (MnP), alkane hydroxylase (AH), catechol 1,2 dioxygenase (C1,2O), catechol 2,3 dioxygenase (C3,2O), and protocatechol 3,4 dioxygenase (P3,4O) presence by searching in the Universal Protein Resource database (UniProt), available at: <https://www.uniprot.org/>, (update 2021/06) (UNIPROT, 2021). Information about the strain code and the names of enzymes and amino acid sequences were collected in order to compare with consortium species. Afterwards, nucleotide sequences were analysed in the National Center for Biotechnology Information database (NCBI), available at: <http://blast.ncbi.nlm.nih.gov/Blast.cgi> (update 2021/06) (20), using BLAST command with standard parameters (ALTSCHUL et al. 1990), following a similarity criterion above 90 % with UniProt-identified strains.

Common ancestor classifications between amino acid sequences were represented in cladograms using the MEGA X - Molecular Evolutionary Genetics Analysis program across computing platforms v.10.2.5 (KUMAR et al., 2018). The evolutionary history was inferred using the unweighted pair group method with arithmetic mean (UPGMA) (SNEATH; SOKAL, 1973), through the ClustalW alignment matrix, and applying 1000 bootstrap replicates to obtain cluster support (FELSENSTEIN, 1985). Similarities between protein groups were plotted on the Venn diagram using a virtual tool of the Bioinformatics & Evolutionary Genomics group, available in<http://bioinformatics.psb.ugent.be/cgi-bin/liste/Venn/calculate_venn.htpl>.

10.4 RESULTS AND DISCUSSION

In this study, an approach was developed to assess a microbial consortium biological phenotype based on its bioremediation capacity and the metabolic state as a functional complement for the analysis of the DNA sequences. As a result, from all 34 microbial consortium strains evaluated in UniProt, a total of 1159 proteins "associated with bioremediation" were foundto be involved with the consortium among the selected groups, representing seven

consortium genera (Table 14), of which 6.7 % (78) belonged to *Bacillus* sp.1, 6.5 % (75) belonged to *Penicillium* sp. 2 and 5.5 % (64) to *Pseudomona*s sp. 2. The taxon with the highest number of enzymes was OH4 and RB4 (*Bacillus* sp.1) with 43 laccase enzymes, 1 alkane hydroxylase enzyme, 2 catechol 1,2 dioxygenase enzymes, 31 catechol 2,3 dioxygenase enzymes, and 1 protocatechol 3,4 dioxygenase, the main enzymes involved in oil degradation. Manganese peroxidase and lignin peroxidase were not found for any of the strains in UniProt, however, there are reports in the literature about extracellular ligninolytic activity in *Aspergillus* (ASEMOLOYE et al., 2018), *Penicillium* (PRENAFETA-BOLDÚ; DE HOOG; SUMMERBELL, 2019), and *Byssochlamys* (M'BAREK et al., 2019) and they are supposedly involved in polycyclic aromatic hydrocarbon oxidation (AYDIN et al., 2017). Most likely, these enzymes have not been sequenced and therefore have not been deposited in this database. The strain S45 (*Allophoma* sp.1) did not show results for any of the selected enzymes, however, Krishnamoorthy et al. (2018) showed a *Phoma tropica* strain (current name *Allophoma tropica*) showed the ability to produce 1.02 $U.mL^{-1}$ laccase for textile effluent dye degradation: Congo red, methyl red, reactive blue and reactive violet with the removal efficiencies of 61.73 %, 81.92 %, 29.5 %, and 23.13 %, respectively. This behaviour was also verified in Debnath et al. (2021), who reported laccase production of 2014,21 $U.mL^{-1}$ from the *Phoma herbarum* strain using industrial agro-waste. According to Ezekoye et al. (2018), *Phoma genus* species are associated with petroleum hydrocarbon degradation and are among the most frequent species in the microbial community during contaminated soil bioremediation processes. Furthermore, they are considered indispensable to the lignin decomposition process due to their high level of occurrence and survivability during the degradation process (PITCHAIRAMU et al, 2008). It is believed that the species in question are less studied for these purposes and possibly the mechanisms related to the degradation of organic compounds may be associated with other groups of enzymes not included in this research.

The most frequent proteins found in this study were dioxygenases, including C1,2O (23.9 %) and P3,4O (20.1 %). These molecules are produced by a wide variety of microorganisms, such as *Bacillus subtilis* (SURENDRA; MAHALINGAM; VELAN, 2017), *Pseudomonas aeruginosa* (LI et al., 2020), *Brevibacillus agri* (YANG et al., 2020), and *Aspergillus fumigatus* (SIVASUBRAMANIAN; NAMASIVAYAM, 2015), often identified as C1,2O producers and *Stenotrophomonas maltophilia*, *Stenotrophomonas rhizophila* (PINSKI; HASTEROK; HUPERT-

KOCUREK, 2020) and *Pseudomonas putida* (UPADHYAY; LALI, 2021) with P3,4O detected activity in agreement with our findings.

Alkane monooxygenase acts on alkane aerobic degradation, transforming them into alcohols through the hydroxylation mechanism (GUERRA et al, 2018). This mechanism is frequently mentioned in the literature and is associated with bacteria, however, in this study, its presence in fungi was associated with cytochrome P450 alkane hydroxylase as a putative enzyme with a homologous function. This fact was also be illustrated in Al-Hawash et al. (2018) which induced alkane hydroxylase in *Aspergillus* sp. reaching a maximum peak of 125.5 μmol.mg^{-1} in presence of *n*-hexadecane.

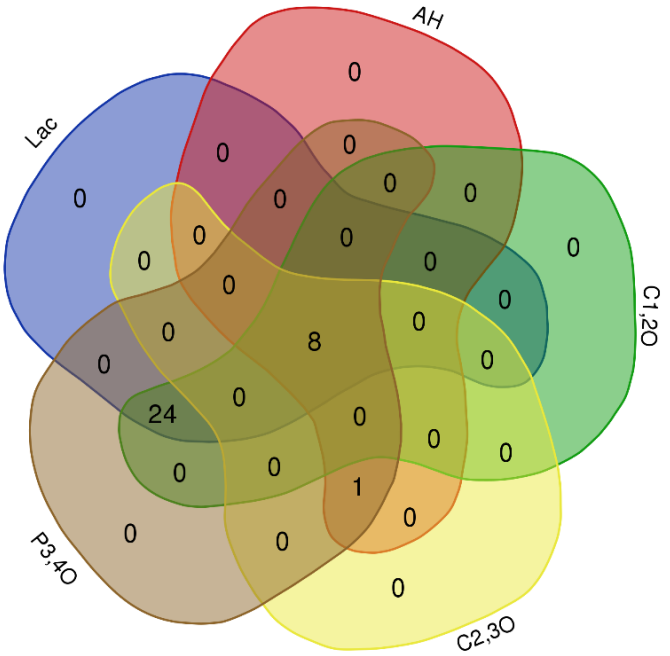
Our results showed that enzyme "meta-analysis" reflects microbiota roles in mangrove bioremediation and the high metabolic exchange occurring between fungi and bacteria. The overlap between different strains according to the five protein approaches is illustrated in the Venn diagram in Figure 30. It was found that 72.7 % of the strains presented similar groups of enzymes, 24.2 % were present at C2.3O and AH and only 3 % were shared between AH, C2,3O, and P3,4O. However, although they are prone to have the same enzymes, they may have different activities and functions in the degradation of organic compounds. In general, laccase participates in oxygen transport, activation, and electron transfer in redox processes. It is known for its multiple functions, such as iron oxidation, CotA, and polyphenols (SUN et al., 2021). In fungi, laccases are involved in lignin degradation, but also nonphenolic substrates, such as aromatic amines, polycyclic aromatic hydrocarbons, synthetic dyes, antibiotics, and other nonobvious laccase substrates, are also involved. On the other hand, although widely discussed, information regarding lignin degradation through bacterial laccases remains unclear and speculative (JANUSZ et al., 2020). The C2,3O enzymes found in this study only among bacterial genera are part of a dioxygenase multicomponent enzymatic system, which is involved in aromatic compound degradation metabolic pathways. In this sense, catechol 2,3-dioxygenase is responsible for the cleavage of the benzene ring in aromatic compounds through the insertion of oxygen molecules, but the reaction is dependent on dihydroxy cyclohexadiene carboxylate dehydrogenase to form catechol (GUERRA et al., 2018). However, even knowing the microorganism enzyme action mechanism, it is difficult to quantify how many proteins are actually used by each species for these functions.

Table 14 - Taxonomic redundancy of strains with similarity to the microbial consortium (BR 10 2021 002341 4) and their respective enzymes investigated in the UNIPROT database

Microorganisms		Enzymes							
		NCBI	UNIPROT						
Code	Species	N° strain**	Lac	MnP	Lip	AH	C1,2O	C2,3O	P3,4O
OH4	*Bacillus* sp.1	99	43*	0	0	1	2	31	1*
RB4	*Bacillus* sp.1	43	43*	0	0	1	2	31	1*
RC5	*Brevibacillus* sp.1	100	0	0	0	1	0	1*	2*
RC6	*Stenotrophomonas* sp.1	51	1	0	0	2	1*	18	4
PD5	*Stenotrophomonas* sp.1	51	1	0	0	2	1*	18	4
PD8	*Stenotrophomonas* sp.1	51	1	0	0	2	1*	18	4
RA2	*Pseudomonas* sp.1	99	1*	0	0	2	1	35*	6
PD6	*Pseudomonas* sp.2	99	1*	0	0	5	6	35*	17
PD7	*Pseudomonas* sp.2	99	1*	0	0	5	6	35*	17
R2	*Aspergillus* sp.1	7	12	0	0	0	29	0	10*
R16	*Aspergillus* sp.1	34	12	0	0	0	29	0	10*
R33	*Aspergillus* sp.2	7	12*	0	0	0	29*	0	10*
S45	*Allophoma* sp.1	8	0	0	0	0	0	0	0
N89	*Byssochlamys* sp.1	20	2*	0	0	0	9*	0	1*
N101	*Byssochlamys* sp.1	20	2*	0	0	0	9*	0	1*
R11	*Penicillium* sp.1	10	4*	0	0	0	8*	0	5*
R26	*Penicillium* sp.1	98	4*	0	0	0	8*	0	5*
R27	*Penicillium* sp.1	57	4*	0	0	0	8*	0	5*
R28	*Penicillium* sp.1	14	4*	0	0	0	8*	0	5*
R30	*Penicillium* sp.1	57	4*	0	0	0	8*	0	5*
S36	*Penicillium* sp.1	87	4*	0	0	0	8*	0	5*
S38	*Penicillium* sp.1	57	4*	0	0	0	8*	0	5*
S39	*Penicillium* sp.1	57	4*	0	0	0	8*	0	5*
S40	*Penicillium* sp.1	57	4*	0	0	0	8*	0	5*
S41	*Penicillium* sp.1	57	4*	0	0	0	8*	0	5*
S52	*Penicillium* sp.1	57	4*	0	0	0	8*	0	5*
A80	*Penicillium* sp.1	51	4*	0	0	0	8*	0	5*
A83	*Penicillium* sp.1	57	4*	0	0	0	8*	0	5*
N102	*Penicillium* sp.1	57	4*	0	0	0	8*	0	5*
R31	*Penicillium* sp.2	19	4	0	0	0	8	0	5*
A79	*Penicillium* sp.2	18	4	0	0	0	8	0	5*
A84	*Penicillium* sp.2	19	4	0	0	0	8	0	5*
N82	*Penicillium* sp.2	39	4	0	0	0	8	0	5*
N96	*Penicillium* sp.2	19	4	0	0	0	8	0	5*

* Results referring to the most similar species within the research genus, since they were not found for the target species of the consortium. ** Taxonomic redundancy found in NCBI referring to species with >95% similarity of up to the first 100 results.
Source: Author, 2022.

Figure 30 - Venn diagram of the amount of enzymes shared between species in the microbial consortium of patent BR 10 2021 002341 4. Lac = Laccase, AH = Alkane hydroxylase, C1,2O = Catechol 1,2 Dioxygenase, C2,3O = Catechol 2,3 Dioxygenase and P3,4O = Protocatechol 3,4 Dioxygenase.

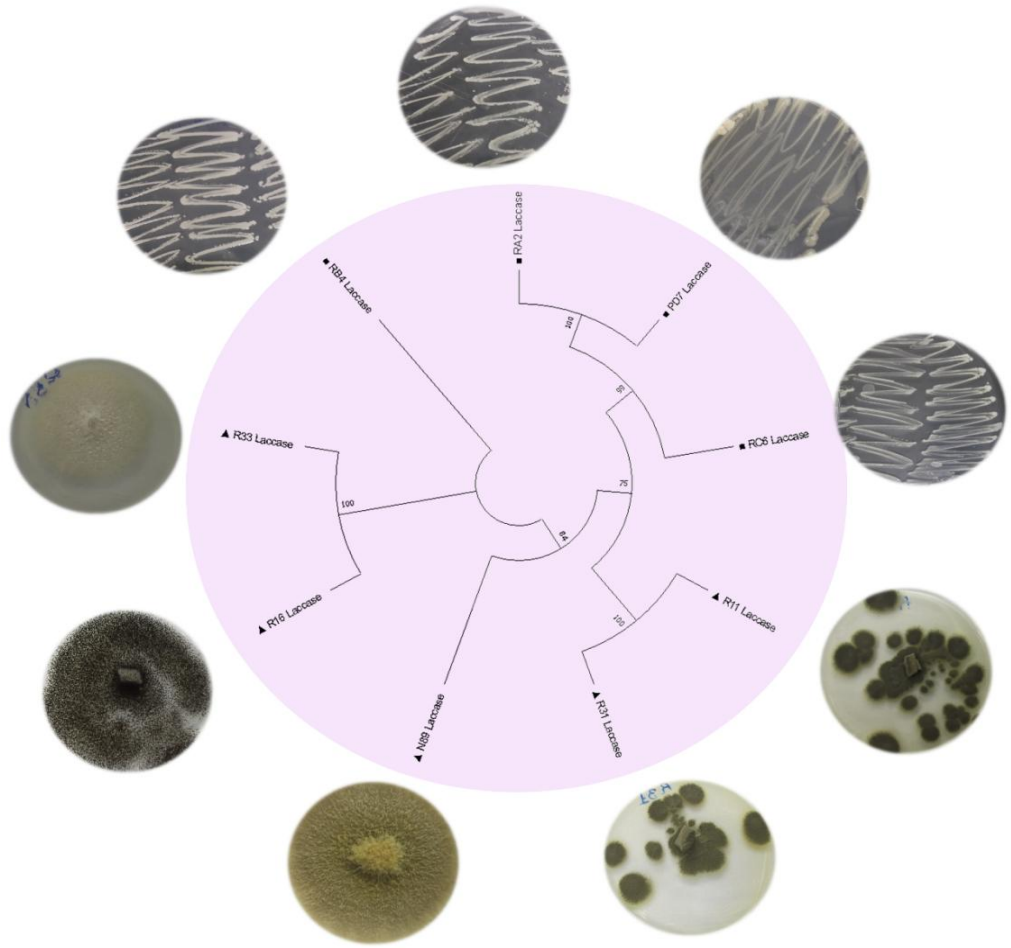


Source: Author, 2022.

In a proteogenomic and/or metaproteomic study, it is not always possible to correlate the microbial population with the protein population, as different species of microorganisms express different proteins under variable environmental conditions. Thus, the in silico analysis has guaranteed value, as it describes the main functions and possible locations of proteins in a given species. For this, calculated distance trees were constructed based on the alignment of the peptides of the strains from the UniProt database (UNIPROT, 2021), with greater similarity to the strains of each genus in the consortium to represent the target enzyme groups, also including the findings of cytochrome P450 alkene hydroxylase between the fungal strains, as shown in Figures 31 and 32. The resulting cladogram shown in Figure 31 can be subdivided into four main clades. The first contains RA2, PD7, RC6, R11, and R31; the second is composed of only N89; the third contains R16, and R33; and the fourth is formed solely by RB4. It is expected that there is low amino acid sequence homology between the fungal and bacterial laccases, however, the molecular conformation is similar, and the general geometry of their active sites is highly conserved (JANUSZ et al., 2020). The fungal strains were concentrated in the centre of the tree, while the bacteria were in the periphery with a greater distance to the *Bacillus* sp. RB4 strain.

Bacteria with laccase activity are recognized as “clandestine proteins”, that is, multifunctional enzymes (JANUSZ et al., 2020). Previous works report that microorganisms of the *Bacillus* genus can act in the degradation of phenols, chlorophenols, compounds derived from lignin (KUMAR; CHANDRA, 2021), agricultural pesticides (GANGOLA et al., 2021), textile dyes congo red, bromophenol blue, violet crystal, and indigo carmine (SHARMA et al., 2021), anionic polyacrylamide (WANG et al., 2021) and reactive black azo dye (SHEELA; SADASIVAM, 2020) in the presence of laccase. Although they are rarely studied among bacteria, their application is currently growing rapidly due to their promising characteristics from an industrial point of view, such as stability over a wide range of temperatures and pH values, wide substrate specificity, short production time, and ease of cloning (CHAUHAN; GORADIA; SAXENA, 2017).

Figure 31 - Cladogram illustrating the distribution of enzymes with similarity within the laccase group. Symbols represent ■bacterial strains and ▲ fungal strains. The purple colour represents laccase.



Source: Author, 2022.

In Figure 32, we can see the grouping of catechol 1,2 dioxygenase with a high similarity between fungi of the same genus and a brief distance from the bacterial strains. However, the strain *Bacillus* sp. RB4 was strain that showed the greatest distance among the others, including among the group of bacteria, assuming that it was probably part of another degradation pathway among dioxygenases. It is known that several bacteria of the *Bacillus* genus are tolerant to the presence of benzene, toluene, ethylbenzene, and xylene (BTEX) and thus have favourable characteristics for the biodegradation of petroleum-derived hydrocarbons (PADILHA et al., 2017). The breakdown of benzene occurs through the mechanism of catechol hydroxylase, starting under the action of catechol 1,2 and later 2,3 dioxygenase. In this process, the (Z)-hex-3-ene dione acid is generated, which is converted into 3-oxoadipate and then results in succinic acid (LI et al., 2021).

Aromatic compounds are metabolized by several microorganisms from intradiol or oestradiol ring cleavage dioxygenases, however, fungi are limited to intradiol cleavage (SEMANA; POWLOWSKI, 2019). Among the 9 sequences of protocatechol 3,4 dioxygenases analysed presented 3 groups with greater distance. The R2 and R33 belonging to the *Aspergillus* genus grouped into a clade sharing similarities to *Penicillium* catechol 1,2 dioxygenase, suggesting that they fall into the intradiol class. Interestingly, the RB4 and RC5 enzymes are allocated to branches from another distant clade, supposedly due to the oestradiol mechanism carried out only by the bacteria. Although all these fungi are able to grow in the presence of petroleum hydrocarbons and related compounds (LIMA et al, 2017), they cannot be associated with the degradation mechanisms exerted by R11, R31, and N89, making evident the distinct spectra within the same group of enzymes.

The comparative phylogenetic analysis of catechol 2,3-dioxygenase showed that there was grouping in the same clade. The similarity between strains that have C2,3O may suggest that aerobic bacteria evolved from the same ancestor and could obtain their dioxygenases by horizontal gene transfer (KOVALEVA; LIPSCOMB, 2007). On the other hand, *Stenotrophomonas* sp. RC6 appeared in another branch close to *Bacillus* sp. RB4, indicating that it has high similarity to the oestradiol dioxygenase ring substrate subfamily, a similar results was found in Wojcieszyńska et al. (2011). Previous work has shown that *Stenotrophomonas* can grow and degrade xenobiotics such as anthraquinone, biphenyl, naphthalene, phenanthridine (ELUFISAN et al., 2020), phenanthrene, fluoranthene, pyrene, total petroleum hydrocarbons,

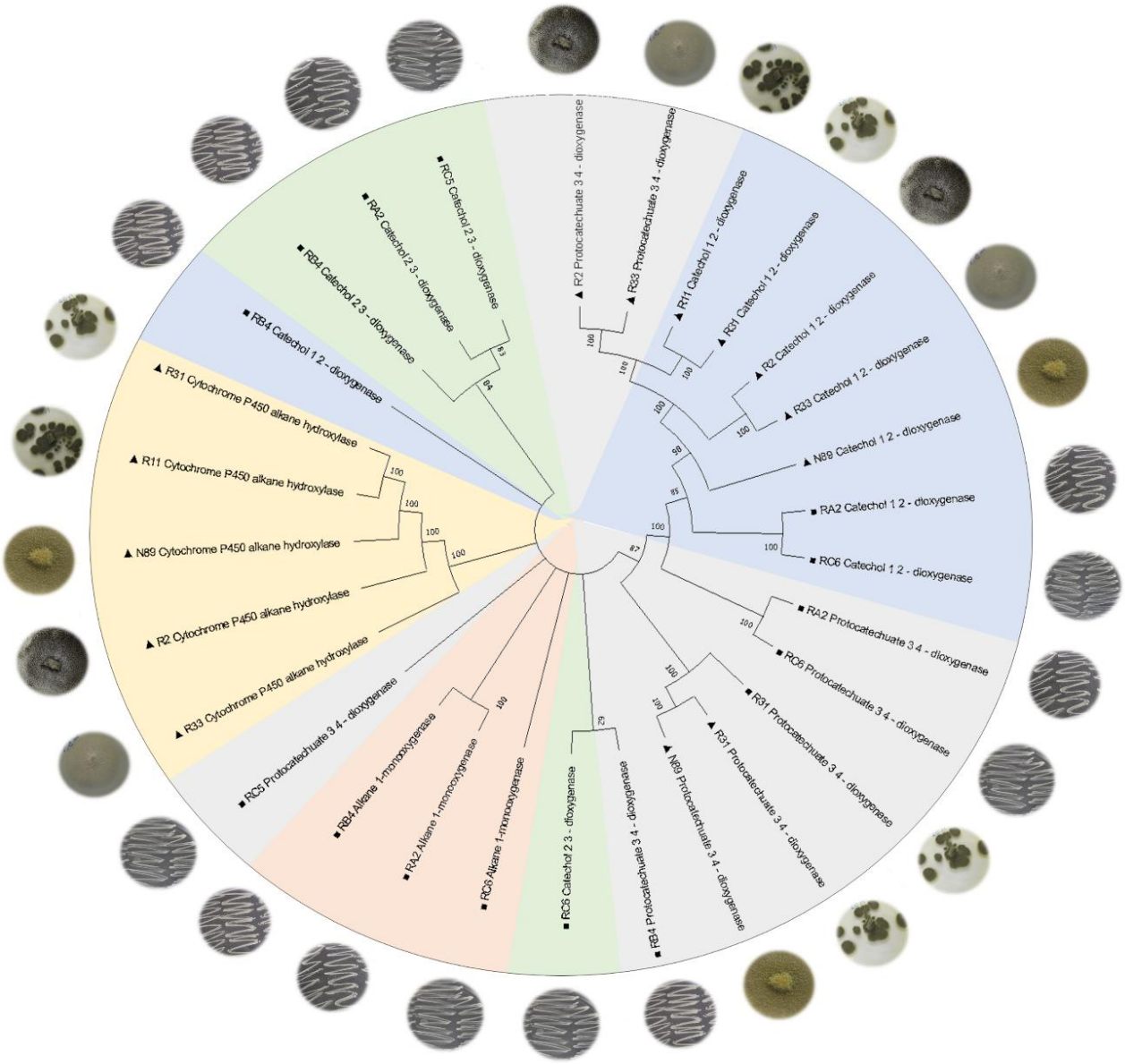
phenolic compounds (PATEL; PATEL 2020), pyrene, benz [a] anthracene, benzo [a] pyrene, dibenz [a, h] anthracene and coronene (JUHASZ; STANLEY; BRITZ, 2000). The degradation effect of these compounds may be associated with the high enzymatic activity of catechol 2,3 dioxygenase described at 61.37 $U.mg^{-1}$ protein in the presence of phenolic compounds (WOJCIESZYŃSKA e et al., 2011).

Alkane hydroxylases have previously been documented to have the ability to mineralize alkanes from short chains (C_2 to C_4) to longer chains (C_{12} to C_{28}), and to act in different patterns of induction (VAN BEILEN et al., 2006; 2003). Thus, the RA2 and RB4 strains, although from different genera, possibly have equivalent functions. The separation of the RC6 strain from the others may be associated with the different degradation mechanisms of alkanes subject to the presence of methane or iron in their degradation pathways (VAN BEILEN et al., 2006). Nevertheless, for CR6, there is a greater similarity of catechol 2,3 dioxygenase to protocatechol 3,4 dioxygenase than its group itself.

Cytochrome P450 (CYPs) are alkane hydroxylases and are ubiquitous in all biological kingdoms, however, their divergence between fungi is multiple, and are involved in the synthesis of degradation of xenobiotic environmental pollutants (DURAIRAJ; HUR; YUN, 2016). In this sense, there is a grouping between the CYPs but with a small diversification in the branches of the tree. It is also notable that although alkane 1-monooxygenases and cytochrome P450 alkane hydroxylase can degrade similar substrates, they have different branches, a fact that leads to belief that the catalytic processes of these fungi and bacteria can be exerted in different degradation pathways.

There is considerable interest in the use of alkane hydroxylases for industrial applications (JI et al., 2013), so the strains studied here can be favourable candidates for biotechnological processes due to the presence of multiple enzymatic systems useful in the biodegradation of petroleum and other recalcitrant compounds.

Figure 32 - Cladogram illustrating the distribution of enzymes with similarity within the group of oxygenases. Symbols represent ■bacterial strains and ▲ fungal strains. The colors refer to: catechol 1,2 dioxygenase in blue, catechol 2,3 dioxygenase in green, protocatechol 3,4 dioxygenase in grey, alkane 1 monooxygenase in orange and cytochrome P450 alkane hydroxylase in yellow.



Source: Author, 2022.

10.5 CONCLUSION

The species in the consortium showed relevance regarding their applicability in the bioremediation of recalcitrant compounds from several enzymatic systems, such as laccase, catechol 1,2 dioxygenase, and protocatechol 3,4 dioxygenase. The functions suggest that, within the same group of enzymes, they can be produced by different species with different mechanisms and, therefore, they can act as a complement to the mineralization of different aromatic compounds.

Although this study was developed for comparisons between the taxon and the number of enzymes associated with oil metabolism, the results obtained demonstrate the applicability of this approach. The results provide the necessary methods to perform semiquantitative comparisons of the composition, physiology and, metabolism of the environmental microbiome as well as a more detailed assessment of microbial composition compared to ITS gene sequencing and 16S rRNA and metabolic functions.

11 OIL DEPLETION INSIGHT FROM MULTIPLE MICROBIAL DEGRADATION SYSTEMS

11.1 ABSTRACT

Petroleum hydrocarbons are organic pollutants that pose a significant threat to various ecosystems and health. Degrading these hydrocarbons through multiple microorganisms is considered an efficient and environmentally friendly strategy. Thus, the objective of the study was to evaluate the degradation of petroleum hydrocarbons by a microbial consortium and to identify which enzymes are differentially expressed during this process. A consortium formed by 33 strains of fungi and bacteria was subjected to a process of oil degradation in liquid medium under agitation at 180 rpm at 35 °C for 7 days. Biodegradation was evaluated for Σ alkanes, resolved hydrocarbons and unresolved complex mixture hydrocarbons by gas chromatography by the adapted protocol from USEPA 3510 and was determined using Agilent MassHunter software. Simultaneously, with the oil depletion process, the enzymatic activity of MnP, LiP, Lac, AH and C2,3O was evaluated in a spectrophotometer, and the enzymes were identified by the shotgun technique in LC–MS-MS with the PatternLab for Proteomics software. The results indicated a decrease in TPH, UCM, Pr, Ph and Σ Alk greater than 40 %. Proteomic analysis confirmed the presence of several enzymes (beta-ketoacyl-[acyl-carrier-protein] synthase, alkyl hydroperoxide reductase C and 2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinol hydroxylase) associated with the degradation of xenobiotics, indicating their possible use in biotechnological applications and in the construction of a remediation library. Finally, the consortium presented promising characteristics for application in hydrocarbon bioremediation processes or equivalents.

Keywords: Whole-cell biocatalyst, bioremediation, microbial consortium, TPH, oil-degrader, proteomic analysis.

11.2 INTRODUCTION

Petroleum hydrocarbons, also known as PHCs, are among the most widespread organic pollutants worldwide and affect multiple ecosystems. Environmental pollution caused by PHCs is responsible for the accelerated flow of hazardous and toxic compounds that pose a significant threat to the ecosphere. Thus, immediate, effective and mainly ecologically viable solutions are urgently needed to address PHCs (DELL'ANNO et al., 2021; ZEHRA et al., 2018).

Crude oil is a complex mixture formed by several hydrocarbons, including paraffins, naphthenes, polycyclic aromatic hydrocarbons, organic sulfur compounds, nitrogen-containing hydrocarbons, phenolic compounds and heavy metals; these hydrocarbons are toxic to humans and other living creatures at certain concentrations (ZEHRA et al., 2018). During the extraction stages, accidents can occur, often resulting from collisions, grounding, structural damage, fires or explosions in transport ships or pipelines. According to the International Tanker Owners

Pollution Federation Limited (ITOPF) database, it is estimated that from 2010 to 2019, approximately 164,000 tonnes of crude oil were spilled into the sea, not including petroleum products. Currently, from 2020 to 2021, approximately 11,000 tons of oil have been spilled (ITOPF, 2021).

Petroleum compounds can be naturally modified or degraded by biotic and abiotic factors. Oil weathering encompasses processes such as evaporation, scattering, adsorption, dispersion, dissolution, emulsion, photooxidation and bioremediation (DE OLIVEIRA, 2020). However, natural attenuation rates can be very slow without the aid of techniques such as bioaugmentation, biostimulation, phytoremediation/rhizoremediation, bioreactors, composting, biopiles, worm remediation, and enzyme-mediated bioremediation (SHARMA, 2022). The numerous technologies available to treat sites contaminated with oil and its derivatives are subject to the structural complexity of the substrate, environmental characteristics, microbial diversity, catabolic potential, operational costs and environmental legislation requirements (RISER-ROBERTS, 1998; CAO et al., 2020). In view of this, bioremediation has shown promising results and has been prevalent in public opinion and regulatory agencies, as it is considered a treatment that involves relatively lower cost, lower energy expenditure and greater probability of environmentally safe results (RAMOS et al., 2016; GURAV et al., 2017; SHARMA et al., 2021).

The degradation of hydrocarbons through the combination of microorganisms (fungi and bacteria) is considered a key component in the remediation strategy (GUPTA; KUMAR; PAL, 2016). It is known that for the complete breakdown of specific pollutants, multiple enzymes are needed, and hardly a single microbial strain is present (DELL'ANNO et al., 2021). Although mixed microbial cultures remain promising in terms of degradation potential compared to that of single cultures, selecting the appropriate microbial consortium requires a thorough understanding of individual performance in the community network (IMAM et al., 2022). Thus, the application of consortia needs to be evaluated as possible bioremediation agents to achieve improved synergistic rates (GUPTA; KUMAR; PAL, 2016). Microbial species have a high capacity to degrade complex compounds due to the activity of enzymes, such as laccase, manganese peroxidase, lignin peroxidase, oxygenase, dehydrogenase, phenoloxidases and others (SHARMA, 2022). The oxidation reduction potential allows these enzymes to degrade a wide range of environmental pollutants, such as dioxins, polychlorinated biphenyls (PCBs), dye effluents, pesticides and petroleum hydrocarbons (BEHNOOD; NASERNEJAD; NIKAZAR, 2014).

Proteomics is a powerful approach to understanding cellular responses to changing conditions through identifying differentially expressed proteins, providing a more comprehensive analysis of the physiological state of cells (CURREEM et al., 2012). Recent advances in high-throughput proteomics techniques have opened new opportunities for understanding the molecular mechanism of biodegradation (MEDIĆ et al., 2019). Although enzymes involved in the degradation of hydrocarbons have already been identified, more research is needed to explore the microbial interactions within the consortium and the mechanisms involved during biodegradation and consequently elaborate more adequate and less invasive strategies in compliance with current environmental laws. Thus, the present work aimed to evaluate the degradation of petroleum hydrocarbons from the action of a microbial consortium and to identify which enzymes are differentially expressed in this process.

11.3 MATERIALS AND METHODS

The consortium of microorganisms used for the oil degradation tests was formed by the following strains: *Aspergillus* sp. (R16), *Allophoma* sp. (S45), *Byssochlamys* sp (N89 and N101), *Penicillium* sp. (R11, R26, R27, R28, R30, R31, S36, S38, S39, S40, S41, S52, S53, A79, A80, A83, A84, N82, N96, N102), *Bacillus* sp. (OH4 and RB4), *Brevibacillus* sp. (RC5), *Stenotrophomonas* sp. (RC6, PD5 and PD8) and *Pseudomonas* sp. (RA2, PD6 and PD7) according to patent BR 10 2021 002341 4 deposited at the National Institute of Industrial Property (INPI) (LIMA et al., 2021). Due to patent protection requirements, the identification sequences of each strain are confidential and are located at the National Center for Biotechnology Information (NCBI) under accession numbers MW865711 to MW865729 for fungi and MW881196 to MV881204 for bacteria. From these microorganisms, the matrix solution of the consortium composed of 1 mL of each preinoculum (10 culture discs dissolved in 0.9 % NaCl and 0.3 % Tween 80) was assembled in a 500 mL Erlenmeyer flask containing 250 mL of Bushnell-Haas (BH) medium (Difco™) supplemented with 1 %(v/v) glycerol (Invitrogen) and homogenized at 180 rpm for 7 days at 30 °C.

The experiment was set up with the addition of a 10 mL aliquot of the standardized microbial consortium matrix solution at 12,089 x 10^3 CFU.mL^{-1} and aseptically transferred to 250 mL Erlenmeyer flasks containing 100 mL of BH mineral broth and 1 mL (± 0.8 g) of oil. The flasks were incubated at 35 °C for 7 days and shaken at 180 rpm using an orbital shaker incubator

(Lab Companion). To prevent oil volatilization and photodegradation, the flasks were sealed with aluminium foil and foil, and the incubator was lined with brown paper. BH medium with the same concentration of carbon source without microorganisms was used to evaluate abiotic weathering, and samples without consortium and without oil were used as controls. The experiment was carried out in biological triplicate and 10% analytical triplicate. The experimental design consisted of 3 samples with oil at 0 days (PT0), 3 samples with oil at 7 days (PT7), 3 samples without intercropping and with oil at 0 days (BRT0), 3 samples without intercropping and with oil at 7 days (BRT7), 2 samples without intercropping and without oil (CT0), and 2 samples without intercropping and without oil (CT7), totalling 16 specimens.

The oil used originated from the Recôncavo Baiano Basin, in which the characteristic features include a high concentration of paraffinic hydrocarbons, a light API degree (35.0) and a low proportion of compounds that contain nitrogen, sulfur and oxygen (GAGLIANONE; TRINDADE, 1988). The samples were generously provided by Transpetro. The extraction of hydrocarbons in the experiment was carried out using the liquid/liquid (L/L) method based on the USEPA 3510 C protocol (EPA, 1996a) and the oil sample using the whole oil method according to the protocol adapted from the Environmental Protection Agency 8270 D and 3540 °C (EPA, 2007, 1996b). The organic extract was extracted with ultrapure dichloromethane (Merck®) and diluted to a final concentration of 0.02 $mg.\mu L^{-1}$ for subsequent injection in a chromatograph. The analysis was performed in a chromatograph with a flame ionization detector (GC-FID) (Agilent®), Model 7890B. Data were analysed using Agilent MassHunter software provided by the manufacturer and calibrated with internal standards of Mix of nC_8 to nC_{40} alkanes (Sigma–Aldrich®).

The analyses to determine the enzymatic activities of laccase (Lac), manganese peroxidase (MnP), lignin peroxidase (LiP), alkane oxygenase (AH) and catechol 2,3 dioxygenase (C2,3O) were performed in a microplate spectrophotometer, LMR Model 96-4 (Loccus) by reading the supernatants at 0 min and after 10 min of reaction. Lac activity was determined through the oxidation of the syringaldazine-based substrate and subsequent formation of quinone (SZKLARZ et al., 1989) at 525 nm wavelength and calculated through the molar absorption coefficient of 65000 $L.M^{-1}.cm^{-1}$. MnP was determined through the oxidation of phenol red (KUWAHARA et al., 1984), and the reading was performed at 610 nm. The molar absorption coefficient considered to calculate the equation was 22000 $L.M^{-1}.cm^{-1}$. LiP was determined from

the oxidation of veratryl alcohol (ARORA; GILL, 2001) at 310 nm and calculated through the molar absorption coefficient of 9300 $L.M^{-1}.cm^{-1}$. The AH activity was measured by the decrease in NADH at 340 nm (LEE et al., 1996), and the molar absorption coefficient used to calculate the equation was 6220 $L.M^{-1}.cm^{-1}$. The activity of C2,3O was measured by oxidation of catechol and formation of hydroxymuconic semialdehyde, and the absorbance was read at 375 nm (BAGGI et al., 1987). The values were submitted to the equation with a molar absorption coefficient of 36000 $L.M^{-1}.cm^{-1}$.

The extraction was carried out using a buffer consisting of 0.7 M sucrose (Exodo), 0.5 M Tris-base (Ludwig Biotec), 0.1 M potassium chloride (Merck®), 0.09 % hydrochloric acid (Merck) (v/v), ethylenediaminetetraacetic acid (EDTA) (Fisher Bioreagents) 0.05 M and 40 mM diothiothreitol (DTT) (LGC Biotechnology) and ammonium acetate precipitation from the modified method of Isaacson et al. (2006). Initially, 100 mL of the sample was centrifuged at 7870 × g for 15 min at 10 °C, and the precipitate was resuspended in 2 mL of extraction buffer. Cell disruption was performed with glass beads (3 mm) under agitation at 30 rpm 3 times for 5 min with an interval of 1 min at 4 °C. It was then centrifuged for 15 min at 76200 x g at 10 °C. After centrifugation, the supernatant was unified with two volumes of ammonium acetate and incubated at -80 °C for 2 h. The precipitate was collected by centrifugation for 15 min at 7870 × g. Therefore, the pellet was washed 3 times with 500 µL of ammonium acetate and then 3 times with 500 µL of 80 % (v/v) acetone (Synth). The sample was dried and resuspended in 200 µL of ammonium bicarbonate (Sigma–Aldrich®) at 50 mM, pH 8.0. Protein concentrations were determined in a spectrophotometer at 595 nm absorbance under Coomassie brilliant blue dye (BRADFORD, 1976), and the quality was observed on an SDS–PAGE gel (LAEMMLI, 1970) using silver nitrate at 20 % (m/v) for revelation.

The samples were submitted to the protocol of digestion of proteins in solution, as described by Cavalcante et al. (2022). Trypsin inactivation was performed with the addition of 5 % (v/v) trifluoroacetic acid and centrifuged at 15,000 × g for 2 min. After enzymatic digestion, the peptides were subjected to desalting in PeptideCleanup C18 Spin columns (Agilent Technologies) and quantified using the Qubit kit (Thermo Fisher). Both procedures were performed according to the manufacturers. The tryptic digestion product (1 µg) was injected into the LC–MS/MS system and then separated from the combination of the A phases (0.1% formic acid in water) and the B phases (0.1 % formic acid in water). formic in acetonitrile) under a

constant flow of 300 $nL.min^{-1}$. Mass spectrometry analyses were performed using an Ultimate 3000 LC liquid nanochromatography system (Dionex) coupled to a Q-Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer (Thermo Fisher Scientific). All parameters used for LC–MS/MS analyses were performed according to Dias et al. (2022). Biological triplicates of the two treatments (T0 oil and T7 oil) were analysed by LC–MS/MS using analytical triplicates. The reagents used in this step were of high analytical purity specific for LC/MS. Protein identification and quantification were performed using the PatternLab for Proteomics software (version 4), available at:< http://www.patternlabforproteomics.org/> and the aforementioned method by Carvalho et al. (2016). The identification of peptides was based on the spectral count of ions (spectrum count) selected at a false discovery rate (FDR) of 1 % (TANG et al., 2008; AGGARWAL; YADAV, 2016). Then, based on the principle of parsimony (ZHANG et al. 2007), the identified peptide sequences were organized into a set of high-confidence proteins.

The generated data were tabulated using Microsoft Excel®, version 2108 of the Microsoft Office 2016® package and submitted to statistical treatment using R and RStudio Software (R CORE TEAM, 2021), version 4.0.5 using heatmaply packages (GALILI et al., 2018), psych (REVELLE, 2021), Rcmdr (FOX; BOLCHET-VALET, 2020) and subsequent production of graphics. For analysis of variance, a p value of ≤0.05 and a confidence level of 95.0 % were adopted.

11.4 RESULTS AND DISCUSSION

As shown in Figure 33, the chromatographic profile of the residual oil after 7 days of incubation reveals a more expressive decrease in the intensity of the peaks between C_{14} and C_{31} from biotic weathering (Figure 33B1), in addition, to demonstrating a reduction in the sets of compounds evaluated was observed. This suggests an initial stage of biodegradation (PETERS, 1993). The GC–MS analysis revealed that in most samples, partial or total loss of C_8 to C_{12} alkanes was observed, resulting in concentrations below the limit of quantification (<LQM); this loss was probably due to evaporation since these alkanes have greater volatility than the other compounds. C_{16}-C_{28} alkanes constituted a large proportion of the alkanes present in the oil (>100 $mg.L^{-1}$), with C_{19} being the dominant group. Thus, the results demonstrated that the oil was dominated by alkanes with higher relative molecular mass. For most saturated hydrocarbons, the consortium exhibited a depletion of >40 %. Categorically, the sum of compounds $\Sigma C_{13\text{-}20}$, $\Sigma C_{21\text{-}30}$

and ΣC_{31-38} showed removals of 50.23 %, 48.82 % and 51.82 %, respectively. The enzyme alkane hydroxylase derived from *Pseudomonas putida* is responsible for the oxidation of short-chain ($C_{3–13}$) or medium- to long-chain C_{10-20} alkanes (TSAI et al., 2017); however, it was not identified in this study (Table 17). Apparently, the compounds were consumed homogeneously, indicating that the consortium exhibited no preference between light or heavy hydrocarbons. It is known that filamentous fungi spend more energy to produce extracellular enzymes and to perform mechanisms of resistance against aggressive environments, which favours the degradation of more complex and toxic organic compounds (WARDLE et al., 2004). Thus, the heterogeneous degradation ability of the microorganisms of the consortium may have provided the breakdown of different compounds, and consequently, the compounds were degraded uniformly. When submitting these data to analysis of variance between the initial and final time, it appears that the p value was <0.05, proving to be significant. Therefore, a hypothesis is that there is a relevant difference between the alkanes (C_8 to C_{40}) both in the biotic experiment (p=0.000169) and in the abiotic experiment (p=2.2e-16) and between them (p= 0.004196).

The total petroleum hydrocarbons (TPH) reached a degradation rate of 50.85 %, the sum of alkanes (Σ Alk) was reduced by 44.65 %, the sum of light alkanes (Σ L Alk) decreased by 50.60 % and heavy alkanes (Σ H Alk) decreased by 49.57 %, indicating that the consortium significantly degraded the compounds. This phenomenon was also reported by Yuan et al. (2018); however, an 81.45 % removal of TPH and > 90 % of alkanes was performed by the fungus-bacteria consortium in 7 days, suggesting that synergistic degradation of crude oil through the consortium may be advantageous for the bioremediation of contaminated sites for oil. With regard to Σ Alk, similar results were found by Dai et al. (2021), in which 60.75 % of the oil (C_{15}-C_{35} alkanes) was degraded in 8 days by a bacterial consortium formed by *Brevibacillus* sp., *Bacillus* sp. and *Acinetobacter* sp. The simultaneous reduction of long chain paraffinic hydrocarbons (C_{18} to C_{35}), pristane (Pr), phytane (Ph) and unresolved complex mix (UCM) was also observed, as well as in our results.

The trend in the biodegradation rate of petroleum hydrocarbons is carried out initially by alkanes > branched alkanes > low molecular weight aromatics > cyclic alkanes, until more complex compounds, such as resins and asphaltenes, are reached in more advanced stages of the process (ROJO et al., 2009). The UCM is mainly composed of branched and cyclic aliphatic hydrocarbons and aromatic hydrocarbons, which are persistent (NIEVAS et al., 2008).

Commonly, the accumulation of compounds in the UCM is reported to be indicative of the oil depletion process; however, its reduction is reflected in a more accentuated degradation. This process is rarely observed in short monitoring periods; however, in this experiment, the UCM decreased by 62 %. In the research by Kim et al. (2022), a reduction in both UCM and TPH from biological treatment was also observed at 90 days, suggesting that the application of the microbial community together with other techniques can be effective in breaking the residual oil.

On the other hand, the abiotic treatment showed negative degradation values, that is, there was an increase in the concentration of dissolved hydrocarbons in the liquid medium after 7 days of experiment. This fact leads us to believe that weathering without the action of the microbial consortium occurred more slowly and that most of the oil probably remained on the surface of the medium. Interestingly, the treatment subjected to the action of microorganisms after seven days presented a cloudy appearance and small miscible droplets as if it had been subjected to a surface-active agent, while the abiotic weathering oil presented itself as larger and more consistent stains on the surface of the medium, shown in Figures 33A4 and B4. Supposedly, this reaction is linked to the behaviour of microorganisms that colonize the surface of the contaminant while secreting macromolecules and extracellular proteins. During this process, depolymerase enzymes together with biosurfactants can be secreted into the extracellular environment and convert hydrophobic substrates into a more hydrophilic surface (KIM et al., 2020).

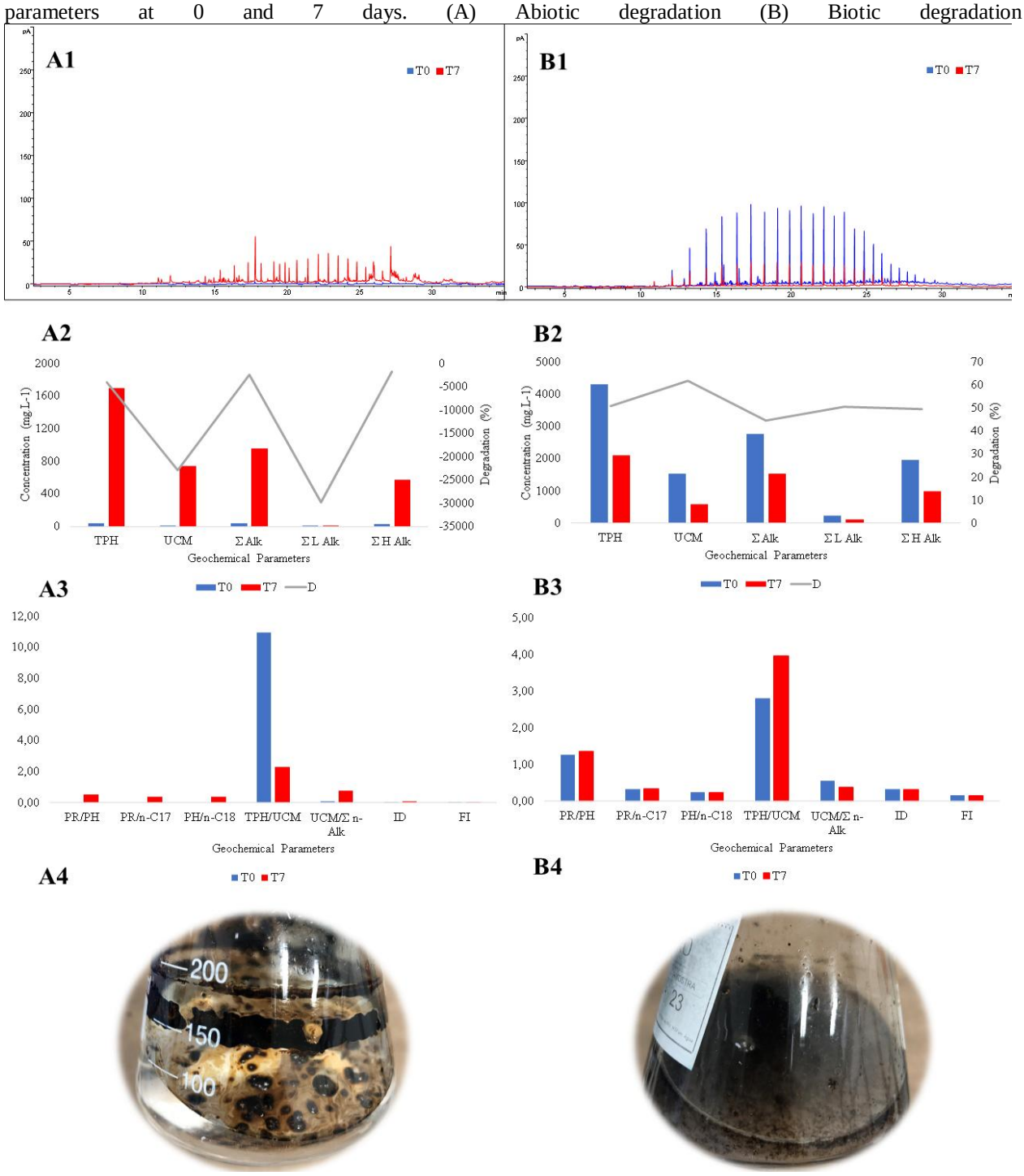
Oil degradation can also be confirmed by several geochemical parameters illustrated in Figures 33 A3 and B3. During the initial biodegradation, a reduction in the TPH/UCM ratio is expected, since lighter hydrocarbons are preferentially removed while molecules more resistant to biodegradation accumulate, forming a hump (KILLOPS; AL-JUBOORI, 1990). Despite this, there was an increase in the TPH/UCM ratio at 7 days of incubation. This fact is probably linked to the simultaneous biodegradation of recalcitrant molecules, such as: compounds containing sulfur and nitrogen, which are essential macronutrients for the development of microorganisms and consequently contribute to the reduction in UCM. The ratio of UCM to Σ alkanes (UCM/Alk) can also be used as a diagnostic criterion for degradation, in which values >10 indicate the presence of weathered oil (SILVA et al., 2013). The values of this ratio in both treatments were <1, which his far below the stipulated criterion. Alternatively, a relatively low value may be indicative of recent entries (TOLOSA et al., 2004), which corroborates the observation period of the experiment.

Since branched alkanes tend to have more inferred degradability compared to that of straight chain alkanes, the isoprenoids pristane (Pr) and phytane (Ph) are considered recalcitrant and therefore were proposed as markers to assess the extent of degradation of spilled oil. (PEREIRA; GOMES; SORIANO, 2009; MODEL et al., 2017). Although isoprenoids are less susceptible to biodegradation, reductions in 47.40 % for Pr and 51.52 % for Ph were observed in this experiment. These values were lower than those found by Dai et al. (2021) in a process of biodegradation of hydrocarbons by bacterial consortium in 8 days (Pr 89.26 % and Ph 77.88 %). Pristane/C_{17} (Pr/C_{17}) and Phytane/C_{18} (Ph/C_{18}) ratios are also widely used in parallel to confirm the degradation of petroleum hydrocarbons (OVERTON et al., 1981; STEINHAUER; BOEHM, 1992; BARAKAT et al., 2001). According to this criterion, the subtle increase observed in the Pr/C_{17} and Ph/C_{18} ratios reveals a tendency towards the reduction of C_{17} and C_{18} alkanes. This behaviour is expected due to the natural inclination of microorganisms to initially consume simpler molecules and conserve isoprenoids, since unbranched hydrocarbons are more easily biodegraded. According to Steinhauer and Boehm (1992), values with ratios < 1 indicate the presence of degraded oil, and values > 1 indicate recent or nondegraded oil. For both concentrations, the values of these ratios were lower than 1, which means that there was oil degradation occurred.

Due to the complexity of oil composition, it is necessary to jointly evaluate other parameters in order to confirm the removal of hydrocarbons. In this sense, there was an increase in the Pristane/Phytane ratio (Pr/Ph), indicating a preference for phytane. Values > 1 are indicative of biogenic origin, and values < 1 suggest petrogenic contribution, that is, moderately biodegradable oils present ratio values higher than those of nonbiodegraded oils. Therefore, the Pr/Ph ratio (1.26 to 1.37) in the biotic treatment confirms the occurrence of the biodegradation process. The weathering index (ID) and the weathering factor (FI) are commonly used to assess oil weathering, as suggested by Wang and Fingas (1995) and Barakat et al. (2001). The even-odd ratio between light and heavy alkanes of the weathering factor remained similar in the sampling times, demonstrating the biodegradation process of both ratios, in with the heavy compounds have a higher concentration than thet of light compounds. This result corroborates the findings regarding the sum of light and heavy alkanes, as well as the other individual degradation parameters. The ID showed a similar behaviour, indicating degradation occurred by biotic action, while the values for the abiotic treatment indicated that the heavy compounds in ID and FI

increased but no change occurred in the lighter compounds. Although the increase in molecules > C_{18} is indicative of degradation, on this occasion, the relationship is not applicable as observed in the other parameters described above.

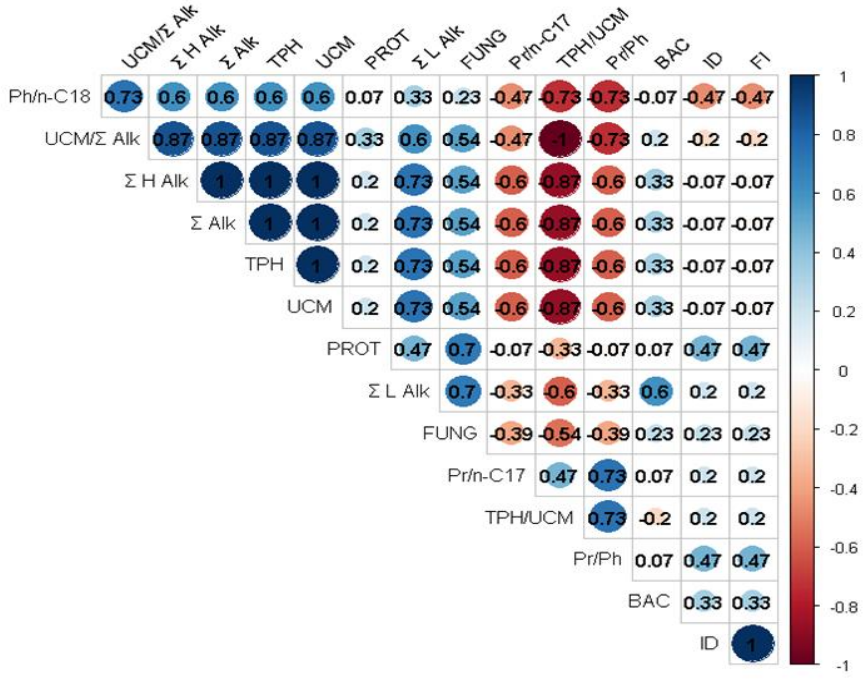
Figure 33 - Chromatographic profile of total petroleum hydrocarbons (C_8 to C_{40}) and geochemical depletion parameters at 0 and 7 days. (A) Abiotic degradation (B) Biotic degradation



Source: Author, 2022.

In Figure 34 shows the correlation matrix between the parameters studied. It was found that there was a strong positive correlation between fungal growth (FUNG), the concentration of light alkanes and total protein (PROT). There was also a moderate positive correlation between fungi and the parameters UCM, TPH, Σ Alk, Σ H Alk and UCM/Σ Alk. For bacteria (BAC), only the correlation between L Alk was observed. Thus, it is concluded that the higher the concentrations of petroleum compounds are, the greater the amount of colony forming units (CFUs) of fungi and bacteria. These results may be associated with the fact that filamentous fungi can transport and adsorb hydrophobic contaminants, such as petroleum hydrocarbons, using them as sources of carbon and, consequently, helping in the degradation rate (SCHAMFUß et al., 2013). Furthermore, the dispersed growth of hyphae can increase accessibility to contaminants for aggregated bacteria (GU et al. 2017). For the other parameters, a strong and very strong correlation between them was already expected, since they are included in the investigated ratios.

Figure 34. Kendall correlation matrix between geochemical degradation parameters and microbiological parameters of biotic weathering



Source: Author, 2022.

It was expected to detect the enzymatic activity of putative enzymes among the microorganisms of the consortium and was cited in the literature as being responsible for the degradation process of petroleum compounds, such as laccase (Lac), manganese peroxidase (MnP), lignin peroxidase (LiP), alkane hydroxylase (AH), and catechol 2,3 oxygenase (C2,3)

(SHARMA, 2022). However, the activity of these enzymes was very low or null. Most likely, the degradation conditions, such as pH, temperature and time, were not favourable to the increase in the activity in question.

With regard to microbial growth during the alteration of petroleum compounds, a decrease in both fungal and bacterial CFUs was observed. This behaviour is common due to the decrease in the biodegradable components of the oil and accumulation of toxic intermediate products generated during the degradation process, causing an inhibition of microbial growth and the metabolic activity of microorganisms (XIA et al., 2019; GUPTA; KUMAR; PAL; 2016). Although the bacterial count is significantly higher than that of fungi, it is not possible to infer that the bacteria are more responsible for the process, since their growth rate is naturally faster than that of fungi. The proportion of total proteins was very similar at both times; however, the subtle reduction after 7 days is consistent with the difference in the proportion of proteins identified, as shown in Table 15.

Table 15. Enzymatic activity, total proteins and microorganism count in the biotic weathering process at 0 and 7 days of incubation

Sample	**Enzymatic activity ($U.L^{-1}$)**					**Total proteins ($\mu g.mL^{-1}$)**	**Identified protein hits**	**Colony Forming Units (UFC mL x $10^{5)}$**	
	Lac	**MnP**	**LiP**	**AH**	**C2,3O**	**PROT**	**HITS**	**FUNG**	**BAC**
PT0	0,157	0,00	0,096	0,00	0,025	247,66	114	0,0005	9,13
PT7	0,345	0,00	1,195	0,00	0,00	245,105	62	0,0002	5,36

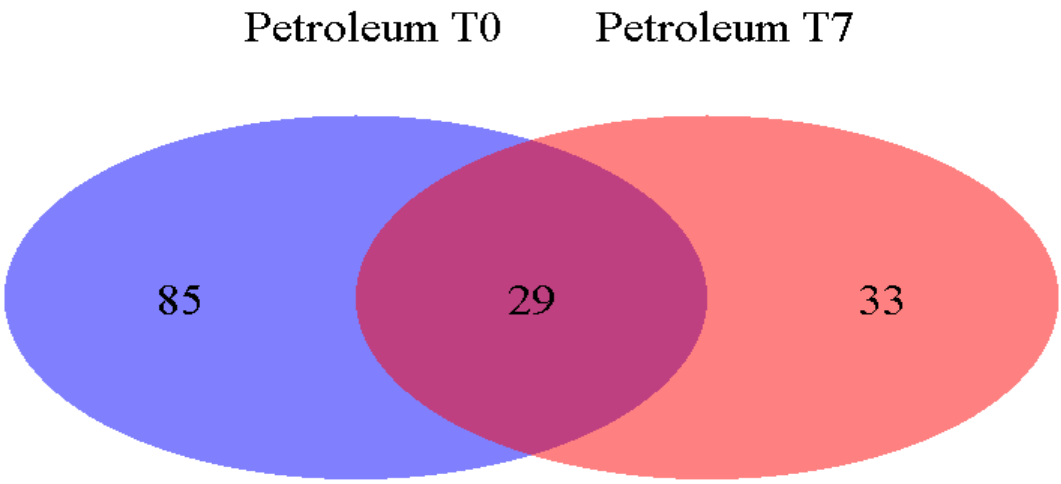
Source: Author, 2022.

A total of 335 protein hits resulted in the identification of 114 total proteins by maximum parsimony and 47 unique proteins at the initial time (T0 Oil), while at the final time (T7 Oil) 62 proteins were identified by parsimony and 25 unique proteins among the 193 hits. proteins revealed (complete data in Appendix 21). In view of these proteomic data, it is noted that there was a reduction of 45.61 % in the total number of proteins identified after 7 days of the experiment. These data corroborate the reduction in the concentration of microbial CFUs, and apparently, this event is related to the toxicity exerted by the petroleum compounds. Similar results were also found by Wang et al (2019) from the identification of proteins in *Rhodococcus* sp. induced by fluoranthene, in which a reduction in 71.57 % of identifications was obtained after 6 days (584 to 166 identified proteins).

When comparing the identification codes (IDs) of the proteins present in the petroleum T0 group with the proteins present in the petroleum T7 group, it was found that 29 proteins were

common in both groups, in which 85 and 33 proteins were identified as exclusive proteins petroleum Groups T0 and T7, respectively (Figure 3). In this scenario, it can be said that 85 proteins are expressed only initially (T0) in the absence of the consortium of microorganisms. On the other hand, 33 other proteins could only be detected after 7 days of exposure to these microorganisms; that is, these 33 proteins were exclusively affected by the exposure of this consortium of microorganisms to petroleum.

Figure 35 - Venn diagram representing the common and exclusive proteins identified in the mixed microbial consortium of patent BR 10 2021 002341 4 in 8000 mg.L^{-1} of oil at 0 (Petroleum T0) and after 7 days (Petroleum T7)



Source: Author, 2022.

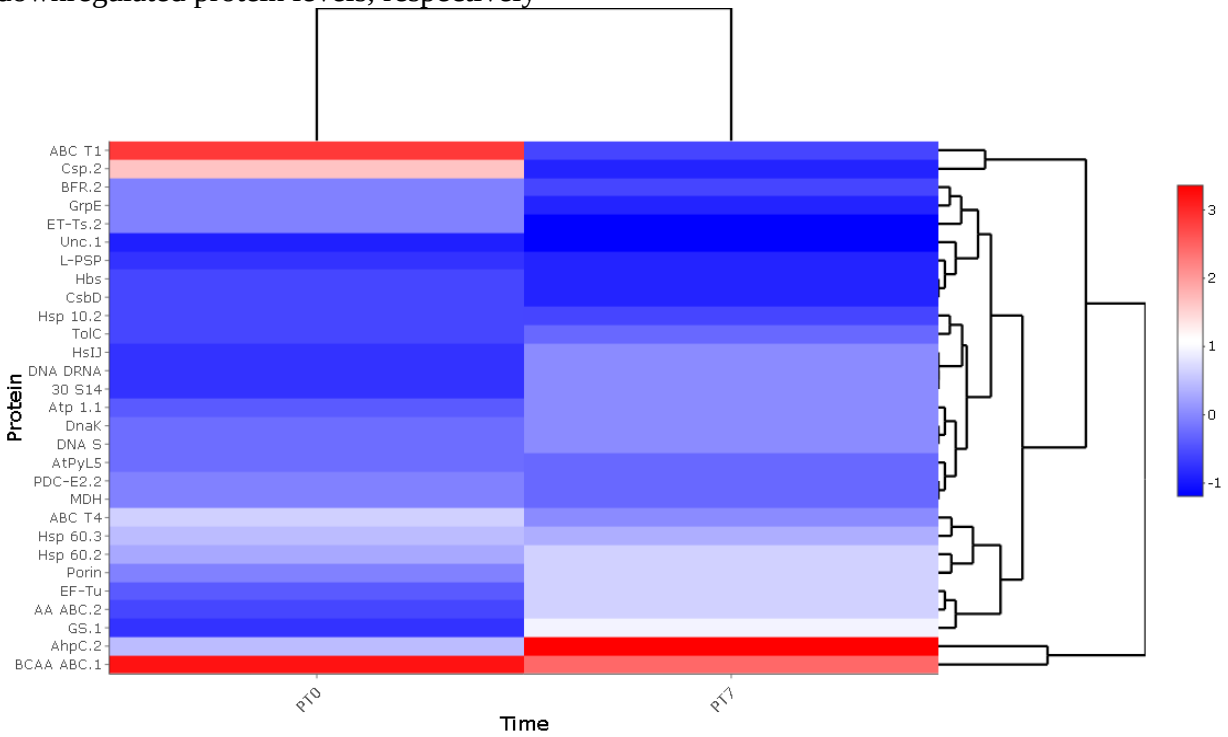
Among the 29 common proteins identified between the two experimental groups, it was observed that these proteins were distributed in 10 functional categories (Figure 36), in which 31.03 % were related to the stress response, 20.68 % were responsible for transport, 20.68 % were involved in translation/transcription, 6.89 % were involved in energy metabolism, 6.89 % were involved in carbohydrate metabolism, 3.44 % were involved in fatty acid metabolism, 3.44 % were involved in metal metabolism, 3,44 % in nitrogen metabolism and 3.44 % were uncharacterized proteins (Table 16).

Considering the fold change values > 1.5, there was a decrease in the abundance of ABC transporter substrate-binding protein, elongation factor Ts, protein GrpE and cold-shock protein, while the protein glutamine synthetase was upregulated between the T0 and T7 groups. Supposedly, these changes in the expression of these proteins are related to the toxicity of the heavier fractions of oil and the need for cells to adapt to the complex carbon source. Of these 29 common proteins, 17.24 % of them showed no change between the two clusters (T0 and T7),

indicating that these proteins may be influenced less by the pollutant in the observation period. The presence of chaperone-like proteins such as: 10 kDa chaperonin, 60 kDa chaperonin, chaperone protein DnaK, CsbD family protein, heat shock protein and protein GrpE both in the common proteome and in the exclusive proteins of the sample groups, perform a task of high importance in the survival and defence mechanism of cells. In this sense, these proteins tend to be more abundant in stressful situations. This mechanism can also occur with proteins of the endoribonuclease type L-PSP, which act as protein synthesis inhibitors (DA FONSECA PIRES et al., 2014).

On the other hand, it appears that most of the common proteins (82.76%) in this study were downregulated. Wang et al. (2019) observed that microorganisms gradually adapt to exposure to hydrocarbons as the only source of carbon and energy, since the concentration of upregulated proteins increases over 8 days of the experiment in relation to that of downregulated proteins. Thus, it is understood that the microorganisms are subjected to an acclimatization step, with a step-by-step change in enzyme richness and diversity.

Figure 36 - Heatmap showing the quantitative differences in common proteins between the Oil T0 and Oil T7 sample groups, in which the red and blue lines represent the upregulated and downregulated protein levels, respectively



Source: Author, 2022.

*The acronyms represent the names of the proteins described in Table 16

Tabele 16 - Common proteins identified in sample Groups T0 and T7 when exposed to a microbial consortium exposed to 8000 mg.L^{-1} of oil from the Recôncavo Baiano basin during seven days of cultivation. Fold change represents the mathematical ratio between protein abundance at T7 in relation to T0

ID Accession (UniProtKB)	Acronyms	Protein Name	Function	Fold Change (Log2)
A0A1U9PSG2	PDC-E2.2	Acetyltransferase component of pyruvate dehydrogenase complex	Carbohydrate metabolism	-0,58
A0A127I331	AhpC.2	Alkyl hydroperoxide reductase C	Carbohydrate metabolism	0,83
A0A1V2K871	Atp 1.1	ATP synthase subunit alpha	Energy metabolism	0,32
A0A127HVG6	MDH	Malate dehydrogenase	Energy metabolism	-0,58
A0A498C516	AtPYL5	Polyketide cyclase/dehydrase/lipid transport protein	Fatty acid metabolism	-0,32
A0A498CTL2	BFR.2	Bacterioferritin	Metal metabolism	-1,00
A0A2S9DEQ0	GS.1	Glutamine synthetase	Nitrogen metabolism	2,00
A0A3N1KFE1	Hsp 10.2	10 kDa chaperonin	Stress response	0,00
A0A1U9Q3S0	Hsp 60.3	60 kDa chaperonin	Stress response	-0,58
A0A3N1KEA9	Hsp 60.2	60 kDa chaperonin	Stress response	-0,19
A0A2S9DFZ4	DnaK	Chaperone protein DnaK	Stress response	0,00
A0A023Y1S8	Csp.2	Cold-shock protein	Stress response	-3,00
A0A3N1K5N7	CsbD	CsbD family protein	Stress response	-0,58
A0A1V2KI48	DNA S	DNA starvation/stationary phase protection protein	Stress response	0,00
A0A498C8D5	HsIJ	Heat shock protein HslJ	Stress response	1,32
A0A2S9DFW8	GrpE	Protein GrpE	Stress response	-1,58
E2RXV3	30 S14	30S ribosomal protein S14	Translation / Transcription	1,32
A0A127HSG9	DNA D RNA	DNA-directed RNA polymerase subunit alpha	Translation / Transcription	1,32
A0A2S9DBZ4	EF-Ts.2	Elongation factor Ts	Translation / Transcription	-2,58
A0A1U9Q4N7	EF-Tu	Elongation factor Tu	Translation / Transcription	0,81
A0A3N1KCE8	L-PSP	Endoribonuclease L-PSP	Translation / Transcription	0,00
A0A3N1KM80	Hbs	Nucleoid protein Hbs	Translation / Transcription	-0,58
A0A1U9PTY3	ABC T1	ABC transporter substrate-binding protein	Transport	-2,94
A0A1V2KIE1	ABC T4	ABC transporter substrate-binding protein	Transport	-1,00
A0A2S9DEL9	AA ABC.2	Amino acid ABC transporter	Transport	1,22
A0A1U9PSP9	BCAA ABC.1	Branched chain amino acid ABC transporter substrate-binding protein	Transport	-0,94
A0A2S9DC43	TolC	Channel protein TolC	Transport	0,42
A0A1U9Q2V0	Porin	Porin	Transport	0,22
A0A3N1KEY1	Unc.1	Uncharacterized protein	Others	0,00

Source: Author, 2022.

The expression profile of differential proteins induced by petroleum from the Recôncavo Baiano basin in a fungal-bacteria microbial consortium is described in Table 17. The 33 enzymes identified differentially after 7 days of the experiment were related to the synthesis of amino acids, carbohydrates, fatty acids and the stress response proteins. It is assumed that these proteins are indirectly involved in the degradation process of the crude oil compounds, since amino acids are essential for the growth and survival of microorganisms (FRANCO; BLANCHARD, 2017), especially in the context of stress caused by the toxic compounds present in petroleum. In addition, it is believed that the identification of proteins related to fatty acid metabolism facilitates the transformation of oil components.

That being said, the metabolism of the oil compounds acting as a support for cell growth is more relevant due to the proteins 4-aminobutyrate-2-oxoglutarate transaminase (GABA-T), aspartate carbamoyltransferase, glycine cleavage system H protein, ketol-acid reductoisomerase and S-adenosylmethionine synthase. GABA-T catalyses the conversion of glutamate to 2-oxoglutarate and plays an important role in crop resistance and stress tolerance. Stress caused by cold, heat, salinity, drought, waterlogging, O_2 deficit, CO_2 accumulation, UV radiation exposure, interactions with fungi and bacteria and other factors can lead to increased GABA-T in cells (SHELP et al., 2017). Zhang et al. (2021) reported a significant increase in GABA-T (EC 2.6.1.19) after the addition of glutaric acid or oxalic acid during increased phenanthrene removal by *Bacillus subtilis*, demonstrating that the addition of these acids can accelerate glutamate catabolism to provide intermediates and energy for cell growth during metabolic processes. In this sense, GABA-T can help in the defence mechanism of cells against exposure to crude oil. In addition to this enzyme, the work also identified ketol-acid reductoisomerase (KARI). KARI acts in the second step of the branched-chain amino acid biosynthetic pathway and can be used for the development of biofuels, herbicides, antibiotics and animal feed (CHEN et al., 2018). In a previous work, this enzyme was identified differentially by Liu et al. (2017), in which the expression of proteins from *Sphingomonas* sp. during the biodegradation of polycyclic aromatic hydrocarbons in the presence and absence of surfactant. In Zhang et al. (2021), KARI was upregulated during the reduction of xenobiotic pollutants by a microbial consortium. Thus, it is believed that these enzymes may be among the main enzymes related to amino acid synthesis during the development of microorganisms in an adverse growth scenario.

Among the group of differential proteins of the T7 group, it was observed that ketol-acid reductoisomerase and S-adenosylmethionine synthase (SAM), superoxide dismutase, bacterioferritin, alkyl hydroperoxide reductase C, glycine cleavage system H protein, 30S ribosomal protein S7 and porin are common to the work of Medic et al. (2019) on *Pseudomonas aeruginosa*-mediated biodegradation of 2,6-di-tert-butylphenol. In addition to the elongation factor Ts, elongation factor Tu, 60 kDa chaperonin, 10 kDa chaperonin, chaperone protein DnaK and ATP synthase subunit alpha proteins were shared between times 0 and 7, indicating that they possibly form a base of aromatic pollutant-associated proteins.

Note the identification of the enzyme beta-ketoacyl-[acyl-carrier-protein] synthase, which may be involved in the production of biosurfactants. One of the main factors limiting the rate of petroleum biodegradation is the difficulty of microorganism access to hydrophobic compounds, and the presence of biosurfactants can increase the bioavailability of most hydrophobic portions of petroleum (SHARMA, 2022). From this wood, this consortium is still relevant due to the possibility of producing biosurfactants affiliated with microorganisms of the *Pseudomonas* genera (BIKTASHEVA et al., 2022), *Bacillus* (SANTOS et al., 2019), *Brevibacillus* (VIGNESHWARAN et al., 2021) and *Stenotrophomonas* (PATEL and PATEL, 2020). In addition, the ability of filamentous fungi to produce biosurfactants was demonstrated by the genera *Phoma* (LIMA et al., 2016), *Aspergillus* (KRELING et al., 2021) and *Penicillium* (MAAMAR et al., 2020). Based on this assumption, the generation of biosurfactants by the microorganisms of the consortium can lead to an improvement in the efficiency of bioremediation by increasing the hydrophobicity and changing the permeability of cell membranes; that is, the biosurfactants helps microorganisms absorb and use hydrophobic compounds (SHARMA, 2022).

The enzyme 2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinol hydroxylase (UbiH) is among the proteins that were differentially identified in the presence of petroleum (T7). The enzyme belongs to the ubiquinone metabolism pathway, which specializes in the catalysis of the oxidation of complex aromatic compounds (UNIPROT, 2022). Furthermore, the biosynthesis of ubiquinone was also considered essential for the denitrification of environments performed by *Pseudomonas aeruginosa* (VO et al., 2020). Ubiquinone was identified in the work of Hou and Majumder (2021) as responsible for the degradation of petroleum-derived compounds, totalling 8018 hits and 4247 genomes. In previous work carried out by Treptow (2018), the expression of

genes encoding UbiH enzymes in *Stenotrophomonas* sp. was examined in the biodegradation of plastics. The biological oxidation of paraffin occurs from the formation of alcohol, followed by the aldehyde being used for the synthesis of fatty acids (CHESHIRE et al., 2012). Therefore, the enzymes that catalyse reactions from these products are part of the "oxidative ladder" that is performed in the bioremediation of sites contaminated by persistent compounds.

Among the strains in the consortium, certain species are reported to produce multiple potential enzymes that degrade petroleum derivatives. The enzymes alpha/beta hydrolase, SGNH/GDSL hydrolase family protein and cytochrome P450 are frequently found in *Bacillus cereus,* and esterase, alpha/beta hydrolase, 4-hydroxybenzoate 3-monooxygenase, cytochrome P450, and alkane 1-monooxygenase are produced by *Pseudomonas aeruginosa* (HOU and MAJUMDER, 2021); however, these enzymes were not identified on this occasion.

Although the identification of proteins in T0 was quantitatively superior in relation to T7, it presented a similar composition in terms of family and function, with the exception of 2,5-diketo-D-gluconic acid reductase, urea ABC transporter substrate-binding protein, pyridoxal phosphate-dependent aminotransferase, protein-export protein SecB, poly(3-hydroxyalkanoate) granule-associated protein PhaF, pirin family protein, P-II family nitrogen regulator, phosphopyruvate hydratase, phospholipid transport system substrate-binding protein, osmotically inducible protein OsmC, NAD(P)-dependent alcohol dehydrogenase, methionine ABC transporter substrate-binding protein, glutamine deaminase RidA, electron transfer flavoprotein subunit alpha, dipicolinate synthase, biotin carboxyl carrier protein of acetyl-CoA carboxylase, asparaginase, acyl carrier protein and FAD-binding oxidoreductase.

Among them, we should highlight the relevance of the enzyme NAD(P)-dependent alcohol dehydrogenase in the transformation of petroleum compounds, in which the mechanism of action plays a central role in bioremediation chemistry. The hydrocarbon degradation pathway uses alcohol dehydrogenase (ADH) to convert the aldehyde corresponding to the alcohol formed in the first step of the pathway by the oxygenase complex protein, and then the aldehyde formed is converted into carboxylic acid (GUENGUERICH; MACDONALD, 1990). Therefore, this enzyme may have participated in the initial process of reducing the hydrocarbons in this experiment. Elumalai and colleagues (2019) performed experiments in which crude oil degradation was induced by the enzyme alcohol dehydrogenase from three bacterial strains isolated; the researchers noted that the intensity of the peaks was considered proportional to the

activity of alcohol dehydrogenase during the degradation of oil between 6 and 10 days. of incubation. ADH biosynthesis was observed by DURÓN-CASTELLANOS et al. (2005) in the first 24 h of incubation in decanate-supplemented medium, followed by a decline together with the growth of *Mucor circinelloides*. This result leads us to believe that ADH was not detected in T7 precisely because of the transformation of the compounds in the initial period of the degradation pathway of aromatic hydrocarbons and/or aliphatic alcohols, as well as the peak phase of consortium growth.

Flavin adenine dinucleotide (FAD)-binding oxidoreductase is involved in the regulation of electrons within groups of dioxygenase enzymes and is part of the mechanisms of anaerobic degradation of aromatic hydrocarbons (BOLL; ESTELMANN; HEIDER, 2020). Cauduro et al. (2017) observed the differential expression of the gene corresponding to this protein after exposure to benzo(a)pyrene.

The presence of thioredoxin at T0 was observed, which plays a key role in maintaining the redox state of thiol. In *Bacillus subtilis* bacteria, TrxA is essential during cell growth and its depletion accompanies a decrease in the growth rate (MOSTERTZ et al., 2008). Thus, the absence of TrxA in T7 together with the other enzymes related to amino acid metabolism corroborate the reduction in CFUs, as seen in Table 15.

The detection of poly(3-hydroxyalkanoate)(PHA) granule-associated protein is an interesting finding because PHA is related to the production of biopolymers used as an alternative for biodegradable plastics, thus adding value to the consortium as a possible bioproduct generator. In addition, the accumulation of PHA is related to the response to poor nutrition and environmental stress, as observed in the experiment (TAN et al., 2019). However, PhaF was absent in T7, assuming that the oil biotransformation process inhibited its perpetuation.

Table 17 - Protein profile of differentially expressed proteins from the microbial consortium exposed to 8000 mg.L^{-1} of oil from the Recôncavo Baiano basin after seven days of cultivation

ID Accession (UniProtKB)	Protein identified	Function	Mol w(Da)*	Spectrum count	Coverage	Genus
A0A1V2K9G8	4-aminobutyrate--2-oxoglutarate transaminase	Amino acid metabolism	44780,2	2	0,0494	*Pseudomonas*
A0A1V2K404	Aspartate carbamoyltransferase	Amino acid metabolism	36238,9	2	0,0569	*Pseudomonas*
A0A498CI70	Glycine cleavage system H protein	Amino acid metabolism	13999,9	1	0,145	*Stenotrophomonas*
A0A127HRK5	Ketol-acid reductoisomerase (NADP(+))	Amino acid metabolism	36170,2	9	0,1361	*Pseudomonas*
A0A2S9DZM8	S-adenosylmethionine synthase	Amino acid metabolism	42523,4	3	0,0455	*Pseudomonas*
A0A2S9DPT3	Citrate synthase	Carbohydrate metabolism	47680,1	2	0,0326	*Pseudomonas*
A0A2S9DPD6	Aconitate hydratase	Carbohydrate metabolism	99133,7	2	0,0186	*Pseudomonas*
A0A1V2JJG8	ATP synthase subunit beta	Energy metabolism	49441,4	6	0,1135	*Pseudomonas*
A0A1V2KIG9	Beta-ketoacyl-[acyl-carrier-protein] synthase I	Fatty acid metabolism	42838,3	1	0,0419	*Pseudomonas*
A0A1V2K7K7	2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinol hydroxylase	Metabolism of aromatic compounds	44588,2	2	0,027	*Pseudomonas*
A0A2S9DRD4	Bacterioferritin	Metal metabolism	17888,1	2	0,0962	*Pseudomonas*
A0A127HV08	Glutamine synthetase	Nitrogen metabolism	51623,5	11	0,0876	*Pseudomonas*
A0A127HXP1	Alkyl hydroperoxide reductase C	Stress response	21901	12	0,16	*Pseudomonas*
A0A2S9E7I9	Trigger factor	Stress response	48416	2	0,0344	*Pseudomonas*
A0A2S9DZR8	YceI domain-containing protein	Stress response	21236,8	4	0,0553	*Pseudomonas*
A0A167YN82	Heat shock protein	Stress response	103377,6	2	0,0139	*Penicillium*
A0A2S9DPQ2	Molecular chaperone HtpG	Stress response	71421,6	2	0,0221	*Pseudomonas*
A0A4S4JEZ9	Peroxiredoxin	Stress response	12304,7	8	0,2054	*Pseudomonas*
A0A1U9Q3G8	Superoxide dismutase	Stress response	21961,8	4	0,1111	*Pseudomonas*

*Predicted molecular weight calculated from the protein sequence. Protein level FDR≤0.01

Source: Author, 2022.

Table 16 - Protein profile of differentially expressed proteins from the microbial consortium exposed to 8000 mg.L^{-1} of oil from the Recôncavo Baiano basin after seven days of cultivation (continued)

ID Accession (UniProtKB)	Protein identified	Function	Mol w(Da)*	Spectrum count	Coverage	Genus
A0A1V2K4E4	50S ribosomal protein L16	Translation / Transcription	15401,6	3	0,1022	*Pseudomonas*
A0A127HSV4	50S ribosomal protein L16	Translation / Transcription	15403,5	2	0,1022	*Pseudomonas*
A0A2S9D3T1	50S ribosomal protein L22	Translation / Transcription	11885,5	2	0,1909	*Pseudomonas*
A0A3N1KJ16	50S ribosomal protein L5	Translation / Transcription	20186,6	2	0,0944	*Stenotrophomonas*
A0A2S9D3R1	50S ribosomal protein L6	Translation / Transcription	19109,3	1	0,0678	*Pseudomonas*
A0A2S9DFU9	Transcription termination/antitermination protein NusA	Translation / Transcription	54433,2	2	0,0345	*Pseudomonas*
A0A2S9E5H2	30S ribosomal protein S6	Translation / Transcription	16257,8	3	0,0714	*Pseudomonas*
A0A1V2K4B4	30S ribosomal protein S7	Translation / Transcription	17618,5	4	0,0769	*Pseudomonas*
A0A2S9D9C3	ABC transporter substrate-binding protein	Transport	28365,8	7	0,1034	*Pseudomonas*
A0A1U9PRV6	Cystine ABC transporter substrate-binding protein	Transport	28357,4	2	0,053	*Pseudomonas*
A0A2S9DI29	Porin	Transport	34537,3	5	0,1012	*Pseudomonas*
A0A2S9DTV0	Putrescine ABC transport system substrate-binding protein	Transport	39925,5	6	0,065	*Pseudomonas*
A0A498CTS0	Uncharacterized protein	Others	30064,9	4	0,1205	*Stenotrophomonas*
A0A2S9D908	Uncharacterized protein	Others	8803,5	2	0,3186	*Pseudomonas*

*Predicted molecular weight calculated from the protein sequence. Protein level FDR≤0.01

Source: Author, 2022.

11.5 CONCLUSION

Insights into HTP degradation help to better clarify microbial performance to design more efficient products in bioremediation, and the combination of microorganisms may offer opportunities for synthetic biology that are not available in pure cultures. That being said, the consortium demonstrated the ability to remove the simplest (50.60%) to the most complex (62%) portion of petroleum hydrocarbons, as confirmed by the main geochemical parameters of biodegradation. Regarding the enzymes found in this study, proteins from amino acid biosynthesis, stress responses and translation/transcription were identified. This set of data contributes to the construction of a remediation library, which makes it possible to effectively apply the microbial consortium as a bioproduct in real sites contaminated with oil or homologous compounds, in addition to providing valuable information on the interactions between bacteria and fungi.

12. MICROBIAL CONSORTIUM PROTEOMIC RESPONSE TO PETROLEUM HYDROCARBONS

12.1 ABSTRACT

The microbial consortium composed of 33 microorganisms from a mangrove environment is capable of growing in a wide variety of petroleum compounds as a source of carbon and energy. As the consortium presents promising characteristics for the degradation of hydrocarbons, a proteomic approach was used to study the enzymes involved in the catabolism of these compounds through the analysis of differentially induced proteins. After 7 days of incubation, the identification of proteins of the fungus-bacteria consortium in the absence and presence of oil was performed using Quadrupole-Orbitrap LC-MS and the evaluation of the concentration of hydrocarbons in a chromatograph with a flame ionization detector. The comparison with the proteome of microorganisms grown in glycerol, the crude oil indicated the induction of enzymes involved in the metabolism of aromatic compounds and fatty acids. The results led to the identification of 76 proteins, where the presence of oil resulted in changes in the abundance of proteins responsible for the oxidative stress response, transport and ribosomal proteins. Concomitantly, there was a reduction of high molecular weight PAHs greater than 40 %, such as: Benzo(K)Fluoranthene, Indene(1,2,3cd)Pyrene and Dibenzo(ah)Anthracene. Therefore, the results can elucidate information about the induced enzymes and provide insight into the optimization of mixed functional consortia with the studied strains for use in the bioremediation process.

Keywords: Biodegradation, bioremediation, HMW PAH, metaproteomics, protein identification, shotgun proteomics.

12.2 INTRODUCTION

As industrialization has advanced, the possibilities of contamination grow and the environment gradually deteriorates. In this context, environmental pollution can reach worrying levels in the future if there is no intervention (LAWAL, 2017). Polycyclic aromatic hydrocarbons (PAHs) have been considered the main environmental villains of public concern due to their deleterious effects and ubiquitous distribution (CUI et al., 2022). PAHs are organic compounds formed by two or more benzene rings fused in various structural configurations (LAWAL, 2017). These hydrocarbons are ubiquitous and are generated in the environment from natural sources or from various human activities, including burning fossil fuels, refining oil, generating electricity, incinerating urban solid waste and oil spills (GUPTA; KUMAR; PAL, 2019). They are hydrophobic pollutants resisting degradation and have harmful biological effects, toxicity, mutagenicity and carcinogenicity, adding relevance to the need for removal (LAWAL, 2017).

Since microbial degradation is quite economical and compatible with the principles of green chemistry, it has been prioritized for environmental remediation in recent decades (GUPTA; KUMAR; PAL, 2016). Consortium-based bioprocesses, in which different specialized microorganisms can efficiently combine various pathways and processes necessary for the degradation of complex substrates, are being developed and investigated as an alternative biotechnological approach (MACCHI et al., 2021). Biodegradation of complex hydrocarbons requires cooperation from more than one microbial species. This is true in the case of pollutants consisting of oil or its derivatives (MOHSIN et al., 2022). Most research on enzymes involved in the catalysis of PAH is focused on a single species of *Pseudomonas aeruginosa* (WANG, QU; SONG, 2021), *Bacillus* sp. (KUREEL et al., 2018), *Rhodococcus* sp. (XU; WANG; KONG, 2018) and others, neglecting information about consortia. However, mixed cultures are considered more efficient in degrading these compounds compared to pure culture (GUPTA; KUMAR; PAL, 2016). For the biotechnological application of consortia, it is necessary to go beyond the confirmation of the efficiency of degradation of undesirable compounds, but also to know the elements involved in the process, such as: enzymes and metabolites (GUPTA; KUMAR; PAL, 2019).

Proteins are the main multifunctional operators in cells, acting as essential structural components, catalysts and key regulatory elements (MARTINS et al., 2019). The study of protein expression has been gradually growing thanks to the proteomics tool and has generated great contributions to the scientific development of several areas of knowledge (DA SILVA-NETO et al., 2019). It is known that a microorganism presents a specific response in the proteome when subjected to a certain carbon source, including organic pollutants (MEDIĆ et al., 2019). Descriptive and quantitative approaches in the identification of a set of proteins, as well as the construction of metabolic pathways for the degradation of recalcitrant compounds, allow us to obtain crucial information for the understanding of variations in the population or changes in response to a given occasion (KHAN, 2018; VALLEDOR; JORIN, 2011). This mechanism of proteome variation combined with information on differential positive or negative regulation of proteins/enzymes under different cultivation conditions can still add reference for the construction of biomarkers and environmental biomonitoring (CHAUHAN; JAIN, 2010; MEDIĆ et al., 2019). In this way, proteomics can generate valuable data to understand the process of

bioremediation of contaminated environments through the identification of proteins responsible for biodegradation (WANG; LI; QU, 2019).

The exploration of omics technology to investigate the degradation processes of polycyclic aromatic hydrocarbons from a microbial consortium would represent a great advance for bioremediation techniques (GUPTA; KUMAR; PAL, 2016). Thus, it would be possible to cross-reference essential information on the degradation of petroleum hydrocarbons, as well as elucidate metabolic pathways in environmental strains and molecular mechanisms of adaptation of natural microbial communities under stress induced by pollutants. In the meantime, the objective of this research is focused on the identification of proteins produced by mixed microbial consortium in the presence of oil from the Recôncavo Baiano basin and its association with the removal of polycyclic aromatic hydrocarbons.

12.3 MATERIAL AND METHODS

The mixed microbial consortium used in this study was isolated from mangrove sediment under laboratory conditions of a bioremediation process (DANTAS, 2016; LIMA et al., 2017). It is formed by 1 microorganism of the genus *Aspergillus*, 1 *Allophoma*, 2 *Byssochlamys*, 20 *Penicillium*, 2 *Bacillus*, 1 *Brevibacillus*, 3 *Stenotrophomonas* and 3 *Pseudomonas*, totaling 33 strains according to the configuration of patent BR 10 2021 002341 4 of the National Institute of Industrial Property (INPI) (LIMA et al., 2021). The solution composed of 1 mL of each inoculum of the consortium was grown in 250 mL of Bushnell-Hass Broth (BH) medium (Difco™) supplemented with 1 % (v/v) of glycerol (Invitrogen) for 168 h at 30 °C with stirring at 180 rpm, as described by Dantas (2022). Then, 10 mL of the matrix solution of the consortium (12,089 x 10^3 CFU.mL) was inoculated into flasks containing 100 mL of BH broth supplemented with oil from the Recôncavo Baiano Basin (8000 mg. L^{-1}) and the control supplemented with glycerol (10 mL.L^{-1}) and kept under stirring at 180 rpm for 168h at 35°C. BH broth grown under the same conditions was used as the experiment blank. Samples and controls were prepared in biological triplicate.

Initially, 100 mL of the sample was centrifuged at 4500 rpm for 15 min at 10 °C and the precipitate was resuspended with 2 mL of extraction buffer consisting of Sucrose (Exodus) at 0.7 M, Tris-base (Ludwig Biotec) at 0. 5M, 0.1M Potassium Chloride (Merck®), 0.09 % Hydrochloric Acid (Merck), 0.05M Ethylenediamine Tetraacetic Acid (EDTA) (Fisher

Bioreagents) and Diothiothreitol (DTT) (LGC Biotechnology) at 40 mM. Cell disruption was performed with glass beads (3 mm) under agitation at 30 rpm for 3 times for 5 min with an interval of 1 min on ice. It was then centrifuged for 15 min at 14000 rpm at 10 °C. After centrifugation, the supernatant was mixed with two volumes of ice-cold ammonium acetate and incubated at -80 °C for 2 h. The precipitate was acquired by centrifugation for 15 min at 14000 rpm. Therefore, the pellet was washed 3 times with 500 µl of ice-cold ammonium acetate and then 3 times with 500 µl of 80 % acetone (Synth). The sample was dried and resuspended in 200 µL of ammonium bicarbonate (Sigma-Aldrich®) at 50 mM, pH 8.0. Protein concentrations were determined in a spectrophotometer at 595 nm absorbance under Coomassie brilliant blue dye (BRADFORD, 1976) and the quality observed on SDS-PAGE gel (LAEMMLI, 1970) using 20 % silver nitrate for development.

The samples were submitted to the protocol of digestion of proteins in solution, as described by Sylvestre et al. (2018). Briefly, the protein solution (50 ug of total proteins) was alkylated in the presence of iodoacetamide and the disulfide bonds were reduced with dithiothreitol, and then subjected to protein hydrolysis by incubation with the enzyme trypsin (1 ng.µL) during overnight at 37 °C. Trypsin inactivation was performed with the addition of 5 % (v/v) trifluoroacetic acid and centrifuged at 15,000 × g for 2 min. After trypsin digestion, the peptides were subjected to desalting on PeptideCleanup C_{18} Spin columns (Agilent Technologies) and vacuum dried according to the manufacturer's protocol.

Mass spectrometry analyzes were performed using an Ultimate 3000 LC liquid nanochromatography system (Dionex) coupled to a Q-Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer (Thermo Fisher Scientific). Ionization was achieved using a Nanospray ion source (PicoChip, Model 1PCH-550, 75 µm ReproSil Pur C_{18} 3 µm; New Objective, USA) with a 2 cm Acclaim PepMap 100 preconcentration trap column (75 µm ID, C18 3 µm; Thermo Fisher Scientific). Six LC-MS runs were performed, consisting of three independent biological replicates of two treatments (oil from the Recôncavo basin and glycerol). Approximately 5 µg of the tryptic digestion product was injected into the system and then separated from the combination of phases A and B. Mobile phase A consisted of 0.1 % formic acid in water and mobile phase B of 0.1 % formic acid. formic acid in acetonitrile under a constant flow of 300 $nL.min^{-1}$. The equipment was set to a gradient of 2 % to 40 % for mobile phase B over 2 hours, followed by a 10 min column wash in 80 % mobile phase B and column reequilibration for 10

min at 2 %. The reagents used in this step were of high analytical purity specific for LC/MS. Sequencing was performed by biological and analytical triplicate.

Protein identification and quantification were performed using the PatternLab for Proteomics software (version 4), available at:< http://www.patternlabforproteomics.org/> and the aforementioned method Carvalho et al. (2016). Peptide identification was based on PSMs (peptide spectrum matching) selected at a FDR (false discovery rate) of 1 %. Then, based on the principle of parsimony (ZHANG et al. 2007), the identified peptide sequences were assembled into a set of high-confidence proteins. To control the rate of false positives at the protein level and assess confidence in peptide identification, a protein FDR of ≤1 % was based on the chosen protein strategy (TANG et al., 2008; AGGARWAL; YADAV, 2016).

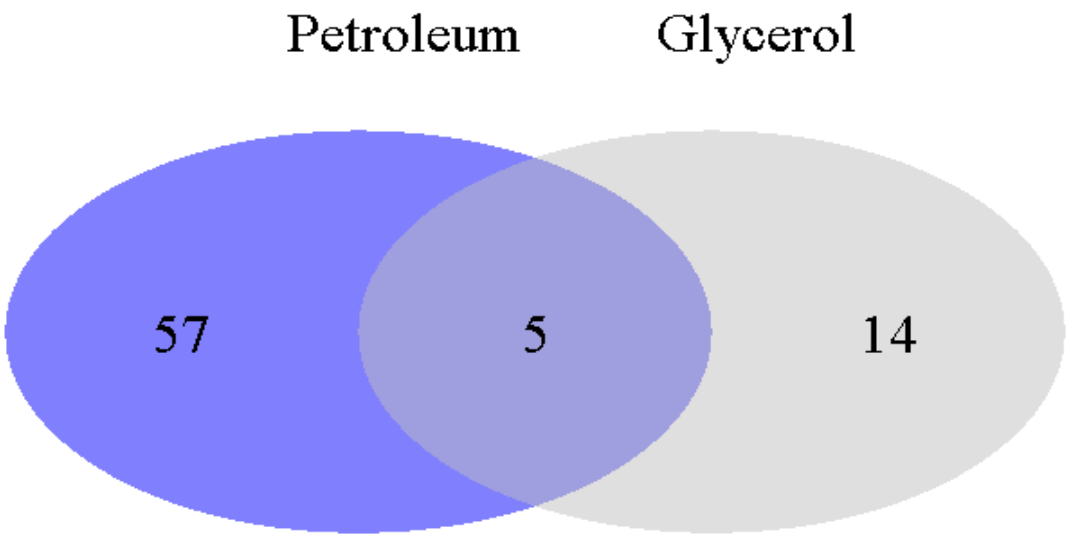
The extraction of hydrocarbons was carried out using the liquid/liquid (L/L) method based on the protocol of the United States Environmental Protection Agency, 3510 C (EPA, 1996a). The organic extract was concentrated and swelled with ultra pure Dichloromethane (Merck®) to a final concentration of 0.02 $mg.\mu L^{-1}$ for subsequent injection into GC-MS. The analysis was performed in a gas chromatograph coupled to mass spectrometry (GC-MS) (Agilent®), model 7890B/5977A, NIST 2011 MS Library (G1033A), using a capillary column DB-5ms, Ultra Inert (5 % phenyl/ 95 % dimethylpolysiloxane, 60m x 250µm x 0.25µm), coupled with a 7693 autosampler, and a 5977A mass detector. With constant flow 1.2052 $mL.min^{-1}$ with carrier gas, Helium. Transferline temperature of 280 ºC. The injection volume was 1 µL, splitless injection mode at 300 ºC. A temperature ramp starting at 55 °C for 1 min, rising at 27°C/min until reaching 300 °C, and holding for 6 min. Samples were acquired in Selective Ion Monitoring (SIM) mode, where the concentrations of the 16 PAHs present on the United States Environmental Protection Agency (US EPA) list of priority pollutants were determined (EPA, 2014). The efficiency of the hydrocarbon recovery rate was evaluated using the p-Triphenyl standard (1000 $ug.L^{-1}$).

The generated data were tabulated using Microsoft Excel®, version 2108 of the Microsoft Office 2016® package and subjected to statistical treatment in Software R and RStudio (R CORE TEAM, 2021), version 4.0.5 using the psych package (REVELLE, 2021), VennDiagram (CHEN, 2018). The fold change was calculated from the formula Log2FC = log2(condition1/condition2). Comparisons between groups were made using the unpaired Wilcoxon test. A significance level of 5 % was adopted, that is, p value ≤0.05.

12.4 RESULTS AND DISCUSSION

The overall proteomic response of the mixed microbial consortium resulted in the identification of 76 proteins, of which 57 and 14 were identified exclusively in cultures enriched with petroleum and glycerol, respectively (Fig. 37). Of all the proteins identified, it was observed that only 5 of the proteins have similarity in common to both conditions and only 1 protein has the same identification, Porin (ID A0A2S9DI29).

Figure 37. - Venn diagram representing the total of proteins identified in the mixed microbial consortium of patent BR 10 2021 002341 4 in 10 mL.L^{-1} of petroleum or glycerol after 7 days



Source: Author, 2022

When comparing all identified proteins in relation to family similarity, it was observed that there is a statistically significant significance between the two groups. Thus, the hypothesis that there is a difference between the count of spectra of the proteins identified in both situations is not rejected. Among the proteins similar to both substrates are Alkyl hydroperoxide reductase subunit C (AhpC), ATP synthase subunit alpha (Atp1), Elongation factor Tu (EF-Tu), Glutamine synthetase (GS) and Porin (Fig. 38). It is believed that the change in the number of spectra of these enzymes is probably due to the effects caused by the xenobiotic compounds present in petroleum.

In comparison with glycerol, the presence of petroleum caused an accumulation of alkyl hydroperoxide reductase, showing a 5-fold increase. It is known that AhpC plays a crucial role in

the defense of cellular components against oxidative damage in bacteria. Its production is associated with a response to high levels of reactive oxygen species (ROS), such as free radicals and peroxides, which can often cause damage to proteins, DNA and cell membranes (JIANG et al., 2019). Upregulation of alkyl hydroperoxide reductase was observed in the decolorization process of Reactive Black 5, Reactive Green 19 and Reactive Red 120 dyes (DE SOUZA et al., 2019), phenolic compound degradation (MEDIĆ et al., 2019), reduction of aromatic compounds (FANG et al., 2019), and in the transformation of phenol and chromium VI (ONTAÑON et al., 2018) and cadmium (IZRAEL-ŽIVKOVIĆ et al., 2018). It is known that the production of ROS is a phenomenon that occurs in parallel with the degradation of PAHs, phenanthrene, flurantene, anthracene, pyrene and benzo[a]pyrene, since their intermediate products such as catechol and quinone play this role (BOURGUIGNON et al. al., 2019). Thus, the high presence of this enzyme can be attributed to the activation of the cellular defense system, which includes the minimization of the generation of ROS, as well as the biodegradation of petroleum compounds.

Adenosine triphosphate (ATP) synthases are protein complexes with several subunits to supply the cell with ATP (GUO, SUZUKI, RUBINSTEIN, 2019). The alpha subunit of ATP synthase involved in metabolism was increased 0.66-fold in response to crude oil. Similar behavior can also be found in the face of phenanthrene exposure in *Amycolatopsis tucumanensis* (BOURGUIGNON et al., 2019). As well, Tiwari et al. (2018) observed a significant change in the transcription levels of ATP synthase α and β subunits in *Fischerell*a sp. after 8 days of pesticide treatment. Other xenobiotic compounds, such as arsenic in *Brevibacterium casei* and cadmium in *Pseudomonas aeruginosa* (SHAH; DAMARE, 2020; IZRAEL-ŽIVKOVIĆ et al., 2018) also showed an increase in ATP beta subunit production. Therefore, possibly this protein may be helping the survival of microorganisms under stress of the contaminant.

It was observed that the Elongation factor Tu had a score 1.75 times higher than glycerol. The same occurred in the research by Bourguignon et al. (2019), where EF-Tu was positively synthesized in cultures exposed to phenanthrene. Wang et al. (2019) revealed an increase (0.58 to 2.17) in the identification of EF-Tu from 1 to 8 days of culture induced by fluoranthene in the growth of *Rhodococcus* sp. Elongation factors participate in protein synthesis, EF-Tu, specifically, is a molecular switch in protein biosynthesis that acts as a carrier of amino acyl-tRNA to the ribosome (WEIJLAND et al., 1992). In this way, the increase in cell replication can be interpreted as necessary to neutralize the oxidative stress generated by oil, as well as to carry

out the metabolism of hydrocarbons (SEO; KEUM; LI, 2009). On the other hand, the negative regulation of this protein indicates that protein biosynthesis is delayed for adaptation to the contaminant (WANG et al., 2019). This information reveals that the elongation factor Tu is closely related to cell growth and that when the protein increases quantitatively in one week, the same occurs with the colony-forming units when exposed to petroleum or its derivatives.

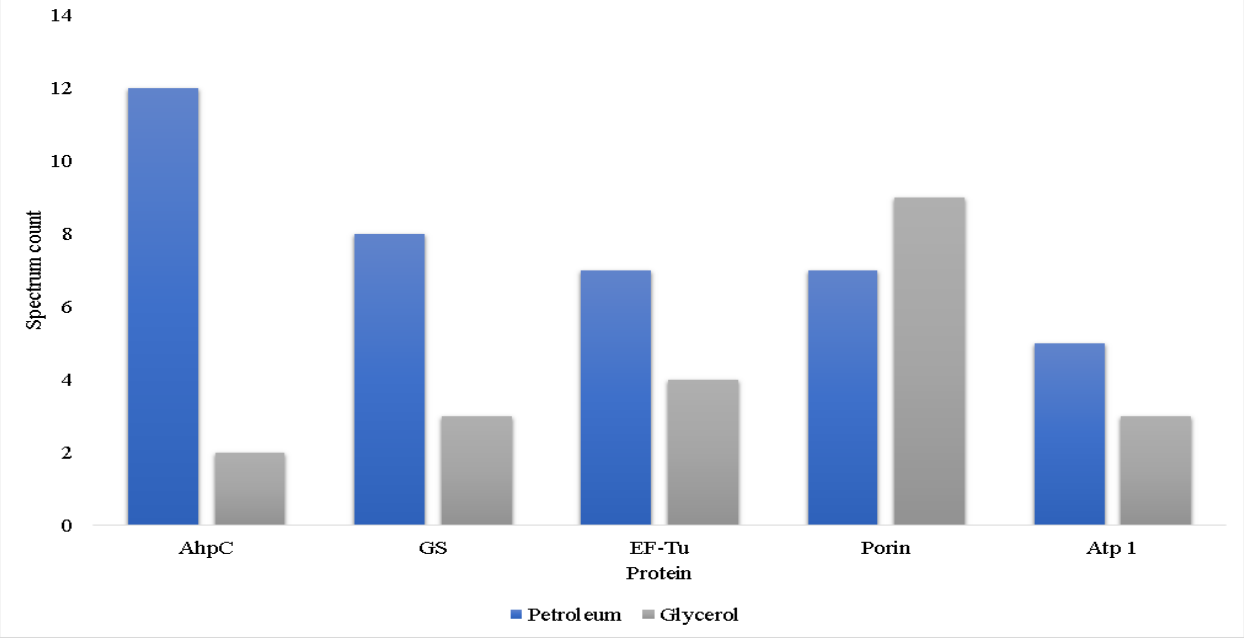
The expression of glutamine synthetase was 2.66 times higher than the control when in the presence of petroleum. Glutamine synthetase (GS) or also known as glutamate-ammonia ligase plays an important role in nitrogen assimilation, as well as ammonia homeostasis (ZHOU et al., 2020). GS converts ammonia into glutamine via ATP-dependent glutamate, reflecting the interaction with carbon metabolism and consequently providing high rates of degradation of petroleum derivatives (CEVHER-KESKIN et al., 2018; GAO et al., 2021). The expression of glutamine synthetase is also associated with the degradation of aromatic compounds, such as aniline (URATA et al., 2004), azo dyes (ZHUANG et al., 2020) and diesel (GAO et al., 2021) corroborating our findings. Interestingly, in the research by Patel et al. (2016) the presence of anthracene and pyrene in the growth of *Anabaena fertilissima* showed a reduction in GS activity with increasing concentrations and over 16 days, indicating exhaustion of energy-producing substrates or direct inactivation of enzymatic complexes. Given the importance of GS participation in carbon metabolism, possibly the increase in GS is related to the catalytic need in the early days of biotransformation of oil components. Since the GS depend on the energy supply, the ATP synthases mentioned above can act in cooperation and provide support to the oil adequacy/depletion mechanism.

In contrast to all other proteins mentioned, porin showed a reduction of 0.22. The same occurred in the study by Tiwari et al. (2017) who noted a decrease in the amount of porins during the degradation of 2-chloro-4-nitrophenol by the *Cupriavidus* strain, suggesting changes in membrane permeability in response to exposure to the aromatic compound. Some porins, such as: OmpD and OmpW are involved in the detoxification of cells through the efflux of toxic compounds, however in terms of stress responses they may grow more slowly due to changes in osmolarity, pH, nutrients and the presence of toxins (DE LA CRUZ; CALVA, 2010). This adaptive mechanism allows the cell to limit the penetration of toxic compounds through the cell membrane (SMULEK et al., 2019). Therefore, its reduction may indicate a response to the

toxicity of petroleum and its metabolites generated in the degradation to control its entry into cells.

Importantly, the presence of petroleum hydrocarbons induces the expression of genes related to stress response mechanisms as the concentration increases (CEVHER-KESKIN et al., 2018). So possibly a higher oil concentration could induce a quantitatively superior identification to the one presented here or the identification of other enzymes related to stress. In general, it is observed that the regulation for both the elevation and the decline of the five proteins mentioned above is linked to the resistance mechanism of microbial strains through the adversities of the carbon source biotransformation process. Likewise, it appears that the structure of the investigated proteome is one of the consequences of the complexity/toxicity of the crude oil compounds, driving further development of enzymes for cell preservation.

Figure 38 - Counting of spectra of similar proteins of the microbial consortium between cultivation in the presence of 10 $mL.L^{-1}$ of oil from the Recôncavo Baiano basin compared to glycerol in seven days of incubation



Source: Author, 2022

The identified proteins were classified into 11 groups, based on their main molecular functions that appeared in the Universal Protein (UniProt) database (http://www.uniprot.org/help/entry_status), National Center for Biotechnology Information: protein (NCBI) (http://www.ncbi.nlm.nih.gov), and PubMed (http://www.pubmed.com). Categories include proteins involved in the metabolism of amino acids, fatty acids, carbohydrates, nitrogen, energy, metals, aromatic compounds, translation and transcription, stress

response, transport and others, uncharacterized, structural and sporulation. Figure 39 shows the distribution of protein composition in relation to function. From the comparison of the profiles and it is observed that the exposure to petroleum hydrocarbons qualitatively and quantitatively altered the patterns. Notably, stress response proteins, along with transport and translation/transcription, accounted for the largest proportion in the presence of petroleum, indicating that they were closely related to adaptation to the carbon source. And it is still possible to notice that the categories metabolism of aromatic compounds and metabolism of fatty acids was only visualized in the sample with oil. On the other hand, most of the fractions detected in the absence of oil are proteins involved in energy metabolism, amino acid metabolism and stress response. Table of Appendix 23 contains the complete list of proteins identified from the microbial consortium in the presence of glycerol.

Among the proteins identified in this study related to stress response are the Cheperones, such as: 10 kDa chaperonin, 60 kDa chaperonin, Chaperone protein DnaK, Molecular chaperone HtpG and other cold and hot shock proteins. The cultivation of microorganisms in aromatic compounds, such as phthalate, can induce the presence of 60 kDa chaperonin, indicating that the increase in this compound induces a stress response (TOMÁS-GALLARDO et al., 2006). The induction of this type of protein was also reported by Medić et al (2019) where it was demonstrated that the use of alkylated phenol 2,6-di-tert-butylphenol differentially regulated the expression of chaperonin compared to the use of sunflower oil. Similar results were also observed by Bourguignon et al (2019), where Cheperones were differentially expressed in the presence of naphthalene, phenanthrene and pyrene in *Amycolatopsis tucumanensis*, suggesting adaptation of cells to exposure to PAHs. Chaperonin expression can also be specifically induced in response to the presence of heavy metals, such as Ni, Co and Cd (CHAKDAR et al., 2022). Vandera et al. (2015) in their analysis found greater abundance of DnaK and GroEL during phenanthrene degradation by *Arthrobacter phenanthrenivorans*. Furthermore, the cheperone protein DnaK has been reported specifically to be associated with cellular stress by aromatic compounds: biphenyl, 4-chlorobiphenyl and 4-hydroxybenzoate in *Pseudomonas* spp. (PARK; OH; KIM, 2001).

Another enzyme identified as in response to stress was superoxide dismutase (SOD), known to participate in intercellular ROS repair and detoxification processes (MITRA et al., 2014). In addition, manganese-dependent SODs are able to act in the significant degradation of organo-solvents, lignin and other aromatic compounds, making them a target of investigation for

biotechnological applications (DIXIT et al., 2021). Wei et al. (2018) detected increased activity of superoxide dismutase in a triphenyl phosphate bioremediation process performed by *Brevibacillus brevis*. Calderón-Delgado, Mora-Solarte and Velasco-Santamaría (2019) reported the same behavior of increase in SOD activity in *Chlorella vulgaris* up to 72 h of cultivation in different concentrations of produced water and crude oil, while Wang and Zheng (2008) reported its induction in *Phaeodactylum tricornutum* when exposed to fluoranthene within 40 h. On the other hand, an increase in SOD expression was observed after 192h in *Fischerella* sp. under stress from methyl parathion, an organophosphate (TIWARI et al., 2018). Since superoxide dismutase is considered a powerful enzyme biomarker (CALDERÓN-DELGADO; MORA-SOLARTE; VELASCO-SANTAMARÍA, 2019), its elevation indicates oxidative stress in the microorganisms of the consortium towards the petroleum compounds during the bioremediation process.

The Ycel protein, despite being identified as an unknown function for several groups of microorganisms, it presents indications of response to osmotic stress with changes in pH and NaCl (ERICKSON et al., 2010; WEBER; KÖGL; JUNG, 2006). Interestingly, the research by Yun et al. (2011) pointed out that Ycel family proteins and ABC transporters showed positive regulation in the cultivation of *Pseudomonas putida* in the presence of benzoate. *Pseudomonas putida* strains are reported to be a source of biodegradable enzymes in response to the presence of aromatic hydrocarbon sources (YUN et al., 2011). However, these reports of detection of this protein under stressful conditions are not conclusive evidence to affirm the function, but provides valuable information that allows us to suggest that it may be related to redox function. Consequently, it is suggested that the protein may have helped to balance the system through the transport and/or storage of amphiphilic compounds (SISINNI et al., 2010).

ATP binding cassette (ABC) transporters are among the proteins with the highest number of spectra in this research (Table 18). In bacteria, ABC transporters have several functions that can be associated with the need to adapt to different environmental conditions (GARMORY; TITBALL, 2004). These mediate the ATP-dependent efflux of endo- and xenobiotic compounds from cells to the external environment and, therefore, become important in protecting the cell against chemical toxicity and stress generated by toxic environmental compounds (NIELSEN; BRODIN; STEFFANSEN, 2010). In addition to acting on the regulation of intracellular levels of ions to macromolecules essential for growth and survival (YU et al., 2015), they import a variety

of allocrits, which were represented in this research by amino acids, cystine, polyamines and other non-specific. Wang, Li and Qu (2019) reported the expression of 16 ABC transporters in crude oil decomposition by *Pseudomonas aeruginosa* and suggested that they are strongly associated with cellular processes related to petroleum hydrocarbon biodegradation. In agreement with Macchi et al. (2021) observed the overexpression of different transport systems, including the ABC transporters (EC 7.4.2.-) related mainly to *Pseudomonas* sp. in bacterial consortium in the degradation of phenanthrene, as well as the increased relative abundance of proteins related to stress resistance.

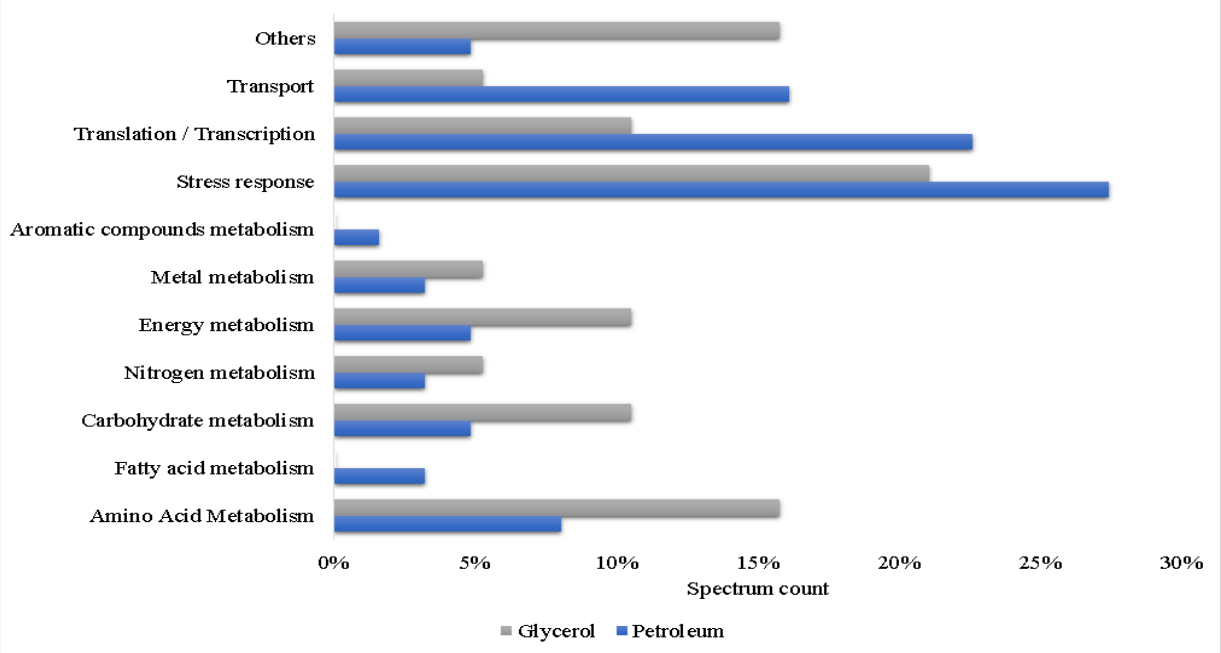
Porins also made up the set of proteins involved in the transport of hydrophobic substrates and the absorption of nutrients. Wang, Li and Qu (2019) observed the induction of the expression of five porins in the presence of petroleum hydrocarbons, while the expression was reduced as the pollutant concentration was increased. This demonstrates that porins play a crucial role in the degradation of petroleum compounds, allowing the flow of hydrocarbons through the cell membrane, however, they may have a limited action according to the toxicity exerted by the concentration of the same. In general, our data corroborate the findings found in previous works related to the degradation of aromatic organic compounds. It appears that in the presence of crude oil there is a dramatic increase in the diversity of proteins associated with homeostasis, where several mechanisms are attributed to overcome the low permeability imposed by the contaminant. The identification of these proteins also corroborates the period of acclimatization of cells to pollution by hydrocarbons, as seen in Dantas (2016).

A list of proteins identified in petroleum-supplemented media during the degradation of petroleum hydrocarbons by mixed microbial consortium is described in Table 18. 193 total hits were found resulting in 62 hits under maximum parsimony. Among them 7 similar proteins: 50S ribosomal protein L16, 60 kDa chaperonin, ABC transporter substrate-binding protein, Alkyl hydroperoxide reductase C, Bacterioferritin, Glutamine synthetase and Porin. It was found that all proteins were categorized into a group of hypothetical proteins. The analysis focused specifically on proteins directly or indirectly related to hydrocarbon metabolism.

Enzymes in the hydrocarbon degradation pathway are often reported to be inducible, as they may have very low or negligible activity in the presence of a single carbon source, such as glucose or organic acids (MOHAPATRA; PHALE, 2021). Based on this assumption, the enzymes Beta-ketoacyl-[acyl-carrier-protein] synthase I, Ketol-acid reductoisomerase

(NADP(+)), S-adenosylmethionine synthase, 4-aminobutyrate--2-oxoglutarate transaminase, Aspartate carbamoyltransferase, Glycine cleavage system H protein, Aconitate hydratase, YceI domain-containing protein, 2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinol hydroxylase and Polyketide cyclase were induced by culturing the consortium microorganisms in solution with petroleum.

Figure 39 - Distribution of the main functional categories of proteins in the microbial consortium in the presence of oil from the Recôncavo Baiano basin compared to glycerol in seven days of incubation



Source: Author, 2022

Proteomic analysis revealed the existence of a representative enzyme of the aromatic ring Hydroxylase subclass. 2-Octaprenyl-3-methyl-6-methoxy-1,4-benzoquinol hydroxylase (UbiH) is among the multiple functional enzymes for the breakdown of petroleum and its derivatives. In agreement with our studies, Peng et al (2018) identified hydroxyl ring dioxygenase genes capable of oxidizing anthracene and benz[a]anthracene from *Rhodococcus* sp. Jin et al (2017) also reported the degradation of HPA (fluoranthene) in the presence of the gene encoding aromatic ring dioxygenase through the *Microbacterium paraoxidans* strain. Hou and Majumder (2021) classified UbiH as having moderate potential for depolymerization of polystyrene, as it has preferential cleavage of the aromatic ring of the styrene group. Therefore, UbiH may also be able to break side chains of petroleum components and oxidize the aromatic compounds generated

from their decomposition and then be processed through microbial energy metabolism until they are mineralized.

The presence of the enzyme Beta-ketoacyl-[acyl-carrier-protein] synthase involved in the biosynthesis of glycolipids that are associated with the production of biosurfactant was observed. The same was reported by Ganesh Kumar et al. (2019) during toluene degradation by *Nesiotobacter exalbescens*. In the work by Elsaeed et al. (2020) the expression of 12 acyl carrier protein (ACP) domains, such as Beta-ketoacyl-[acyl-carrier-protein] synthase, was observed in a sample from a marine ecosystem contaminated by chronic oil pollution and PAH accumulation. The biosurfactant synthesis performed by this enzyme qualifies it as essential for the biodegradation of complex hydrocarbons, since it can act to increase the solubility and biodegradability of these compounds.

Another protein presented was ketol-acid redutoisomerase, a type of oxidoreductase that participates in the biosynthesis of branched-chain amino acids. In the study by Shuona et al. (2017) this enzyme was upregulated in a system co-contaminated with benzo[a]pyrene and copper in a bioprocess mediated by *Stenotrophomonas maltophilia*. Increased expression was also observed in the presence of phenanthrene by Zhang et al. (2021).

The authors pointed out that amino acids are essential for the growth of microorganisms and thus, their biosynthesis contributes to the efficiency of hydrocarbon gradation. Although the species that make up the consortium are reported in the literature as producing enzymes involved in the degradation of petroleum or homologs, the presence of several proteins that are directly involved in the transformation of these compounds was not observed (complete list of petroleum-related proteins in Appendix 15). Possibly, the conditions of cultivation or extraction of proteins have not favored the determination of more specific components for this objective.

Concomitantly with the process of identifying the proteins of the consortium, the concentrations of polycyclic aromatic hydrocarbons (PAH) present in the culture system of the consortium in the presence of oil were also observed. In view of this, the alteration of the main PAHs from the background sample to the experiment in 7 days was obtained, as shown in Figure 40.

Table 18 - Protein expression profile of the microbial consortium exposed to 8000 mg.L^{-1} of oil from the Recôncavo Baiano basin during seven days of cultivation

ID Accession (UniProtKB)	Function	Protein identified	Mol w(Da)*	Spectrum count	Coverage	Genus
A0A1V2KIG9	Fatty acid metabolism	Beta-ketoacyl-[acyl-carrier-protein] synthase I	42838,3	1	0,0419	*Pseudomonas*
A0A498C516		Polyketide cyclase/dehydrase/lipid transport protein	39305,3	4	0,0808	*Stenotrophomonas*
A0A1V2K7K7	Metabolism of aromatic compounds	2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinol hydroxylase	44588,2	2	0,027	*Pseudomonas*
A0A127HVG6	Stress response	Malate dehydrogenase	45039,3	4	0,0569	*Pseudomonas*
A0A4S4JEZ9		Peroxiredoxin	12304,7	8	0,2054	*Pseudomonas*
A0A3N1KFE1		10 kDa chaperonin	9969,3	3	0,2421	*Stenotrophomonas*
A0A1U9Q3S0		60 kDa chaperonin	56952,7	6	0,0894	*Pseudomonas*
A0A3N1KEA9		60 kDa chaperonin	57394,9	7	0,031	*Stenotrophomonas*
A0A127HXP1		Alkyl hydroperoxide reductase C	21901	12	0,16	*Pseudomonas*
A0A127I331		Alkyl hydroperoxide reductase C	20420,4	16	0,2941	*Pseudomonas*
A0A2S9DFZ4		Chaperone protein DnaK	68348,3	5	0,0627	*Pseudomonas*
A0A023Y1S8		Cold-shock protein	7412,8	2	0,209	*Stenotrophomonas*
A0A3N1K5N7		CsbD family protein	8504,1	2	0,1644	*Stenotrophomonas*
A0A1V2KI48		DNA starvation/stationary phase protection protein	17750,1	5	0,2293	*Pseudomonas*
A0A167YN82		Heat shock protein	103378	2	0,0139	*Penicillium*
A0A498C8D5		Heat shock protein HslJ	29666,7	5	0,0985	*Stenotrophomonas*
A0A2S9DPQ2		Molecular chaperone HtpG	71421,6	2	0,0221	*Pseudomonas*
A0A2S9DFW8		Protein GrpE	20729,4	2	0,0753	*Pseudomonas*
A0A1U9Q3G8		Superoxide dismutase	21961,8	4	0,1111	*Pseudomonas*
A0A2S9E7I9		Trigger fator	48416	2	0,0344	*Pseudomonas*
A0A2S9DZR8		YceI domain-containing protein	21236,8	4	0,0553	*Pseudomonas*

Source: Author, 2022.

Table 18 - Protein expression profile of the microbial consortium exposed to 8000 mg.L^{-1} of oil from the Recôncavo Baiano basin during seven days of cultivation (continuation)

ID Accession (UniProtKB)	Function	Protein identified	Mol w(Da)*	Spectrum count	Coverage	Genus
A0A1V2K9G8	Amino Acid Metabolism	4-aminobutyrate--2-oxoglutarate transaminase	44780,2	2	0,0494	*Pseudomonas*
A0A1V2K404		Aspartate carbamoyltransferase	36238,9	2	0,0569	*Pseudomonas*
A0A498CI70		Glycine cleavage system H protein	13999,9	1	0,145	*Stenotrophomonas*
A0A127HRK5		Ketol-acid reductoisomerase (NADP(+))	36170,2	9	0,1361	*Pseudomonas*
A0A2S9DZM8		S-adenosylmethionine synthase	42523,4	3	0,0455	*Pseudomonas*
A0A127HV08	Nitrogen metabolism	Glutamine synthetase	51623,5	11	0,0876	Pseudomonas
A0A2S9DEQ0		Glutamine synthetase	51695,5	8	0,0876	Pseudomonas
A0A1V2K871	Energy metabolism	ATP synthase subunit alpha	55240,6	5	0,0525	Pseudomonas
A0A1V2JJG8		ATP synthase subunit beta	49441,4	6	0,1135	Pseudomonas
A0A3N1KCE8	Translation / Transcription	Endoribonuclease L-PSP	13675	2	0,1154	Stenotrophomonas
A0A1V0B4L3		30S ribosomal protein S6	16257,8	3	0,0714	Pseudomonas
E2RXV3		30S ribosomal protein S14	11409,2	5	0,1287	Pseudomonas
A0A1V2K4B4		30S ribosomal protein S7	17618,5	4	0,0769	Pseudomonas
A0A127HSV4		50S ribosomal protein L16	15403,5	2	0,1022	Pseudomonas
A0A1V2K4E4		50S ribosomal protein L16	15401,6	3	0,1022	Pseudomonas
A0A2S9D3T1		50S ribosomal protein L22	11885,5	2	0,1909	Pseudomonas
A0A3N1KJ16		50S ribosomal protein L5	20186,6	2	0,0944	Stenotrophomonas
A0A2S9D3R1		50S ribosomal protein L6	19109,3	1	0,0678	Pseudomonas
A0A2S9DBZ4		Elongation factor Ts	30538,3	1	0,0418	Pseudomonas
A0A1U9Q4N7		Elongation factor Tu	43535,2	7	0,0453	Pseudomonas
A0A127HSG9		DNA-directed RNA polymerase subunit alpha	36605,3	5	0,1862	Pseudomonas
A0A3N1KM80		Nucleoid protein Hbs	9265	2	0,1667	Stenotrophomonas
A0A2S9DFU9		Transcription termination/antitermination protein NusA	54433,2	2	0,0345	Pseudomonas

Source: Author, 2022.

Table 18 - Protein expression profile of the microbial consortium exposed to 8000 mg.L^{-1} of oil from the Recôncavo Baiano basin during seven days of cultivation (continuation)

ID Accession (UniProtKB)	Function	Protein identified	Mol w(Da)*	Spectrum count	Coverage	Genus
A0A1U9PSG2	Carbohydrate metabolism	Acetyltransferase component of pyruvate dehydrogenase complex	56325,1	4	0,0543	*Pseudomonas*
A0A2S9DPD6		Aconitate hydratase	99133,7	2	0,0186	*Pseudomonas*
A0A2S9DPT3		Citrate synthase	47680,1	2	0,0326	*Pseudomonas*
A0A2S9DRD4	Metal metabolism	Bacterioferritin	17888,1	2	0,0962	*Pseudomonas*
A0A498CTL2		Bacterioferritin	21693,8	3	0,0928	*Stenotrophomonas*
A0A1U9PTY3	Transport	ABC transporter substrate-binding protein	27435,1	3	0,0502	*Pseudomonas*
A0A2S9D9C3		ABC transporter substrate-binding protein	28365,8	7	0,1034	*Pseudomonas*
A0A1V2KIE1		ABC transporter substrate-binding protein	28288,7	5	0,0766	*Pseudomonas*
A0A2S9DEL9		Amino acid ABC transporter	27719,1	7	0,152	*Pseudomonas*
A0A1U9PSP9		Branched chain amino acid ABC transporter substrate-binding protein	39984,4	13	0,0794	*Pseudomonas*
A0A2S9DC43		Channel protein TolC	50194,3	4	0,0377	*Pseudomonas*
A0A1U9PRV6		Cystine ABC transporter substrate-binding protein	28357,4	2	0,053	*Pseudomonas*
A0A1U9Q2V0		Porin	46884,2	7	0,0694	*Pseudomonas*
A0A2S9DI29		Porin	34537,3	5	0,1012	*Pseudomonas*
A0A2S9DTV0		Putrescine transport system substrate-binding protein	39925,5	6	0,065	*Pseudomonas*
A0A2S9D908	Others	Uncharacterized protein	30064,9	4	0,1205	*Pseudomonas*
A0A3N1KEY1		Uncharacterized protein	8803,5	2	0,3186	*Stenotrophomonas*
A0A498CTS0		Uncharacterized protein	10404	1	0,1176	*Stenotrophomonas*

*Predicted molecular weight calculated from the protein sequence. Protein level FDR≤0.01

Source: Author, 2022

From the variations of percentage reduction in the concentration of PAHs observed in Figure 4B, it is possible to divide the compounds into four groups: <10 % represented by Phenanthrene (PHN), Benzo(b)Fluoranthene (BBF) and Benzo(a)Pyrene (BAP); >10<20 % where are Benzo(ghi)Perylene (BGP),Crysen (CHY), Benzo(a)Anthracene (BAA); >20<40 % with Anthracene(ANT), Pyrene (PYR) and Acenaphthene (ACN) and >40<60 % larger group formed by Naphthalene (NAP), Fluoranthene (FLT), Benzo(K)Fluoranthene (BKF), Indene(1,2,3cd)Pyrene (ICP) and Dibenzo(ah)Anthracene (DBA). In the backgroud sample, the compounds with maximum and minimum values were Phenanthrene (510.15 $\mu g.L^{-1}$) and Indene(1.2.3cd) Pyrene (1.14 $\mu g.L^{-1}$), respectively. After seven days of experiment these compounds increased to 490.72 $\mu g.\ L^{-1}$ and 0.51 $\mu g.\ L^{-1}$, respectively. Kumari, Regar and Manickam (2018) were successful in degrading several PAHs from crude oil, such as: 89.1% naphthalene, 63.8% fluorene, 81% phenanthrene and 72.8% benzo(b) fluoranthene from a bacterial consortium consisting of *Stenotrophomonas maltophilia, Ochrobactrum anthropi*, *Pseudomonas mendocina*, *Microbacterium esteraromaticum* and *Pseudomonas aeruginosa* in 45 days. Thus, it is believed that an adjustment in the consortium arrangement proposed here to achieve efficient synergism and coordinated metabolic activities, as well as a longer experiment time could provide efficiency in the degradation rate of aromatic compounds.

Based on the total concentration of the 16 priority PAHs (∑16HPA), it is noted that there was a change of only 7.12 % at the end of seven days (from 787,285 to 731,208 $\mu g.L^{-1}$). By submitting these data to analysis of variance between the background sample and after seven days, it appears that the p-value was higher than the adopted significance level. Therefore, the assumption that there is a relevant change in the sum of PAHs in this study condition is rejected. These findings corroborate the lack of identification of specific enzymes commonly related to hydrocarbon degradation, such as: laccase, manganese peroxidase, catechol 1,2 oxygenases, protocatechol 3,4 dioxygenase, alkane hydroxylase, cytochrome P450, among others. Taking into account that PAHs have a more complex chemical structure and, therefore, are less susceptible to biodegradation than aliphatic hydrocarbons, it is believed that the reduction of crude oil compounds has occurred preferentially among alkanes than PAHs. In addition, the simultaneous occurrence of hydrocarbon alkanes, alkenes, phenols and trace metals present in petroleum can prolong the resistance time of PAHs due to the depletion of available oxygen for the degradation process (ALEGBELEYE; OPEOLU; JACKSON, 2017). Therefore, the significant change in

petroleum degradation is likely to be seen in total petroleum hydrocarbons and for PAHs it may be necessary to investigate the optimal depletion point.

It can be seen that the lighter compounds among the PAHs (2-3 rings) showed a change of 4.21 % in concentrations from 639.02 to 612.09 $\mu g.L^{-1}$. The most complex compounds (4-6 rings) also had their concentrations modified (from 148.25 to 119.11 $\mu g.L^{-1}$) representing a decline of 19.65 %, which indicates that the degradation rate of the most complex compounds was higher, such as Dibenzo(ah)Anthracene with a reduction of 58.59 % and Indene(1,2,3cd)Pyrene with a reduction of 55.13 % (Fig 40B). Low molecular weight (LMW) PAHs are expected to be degraded faster than high molecular weight (HMW) PAHs, as they are more bioavailable due to greater ease of solubilization and volatilization (GUPTA; KUMAR; PAL, 2019), however, in this experiment, the highest reduction rates, greater than 50 %, were observed among HMW PAHs, such as: DBA, ICP and BKF. Although the preferential use of simple carbon sources over complex ones is common, there are strains with mechanisms capable of preferentially consuming aromatic substrates to glucose and co-metabolism of aromatics with organic acids, such as *Pseudomonas putida* (BASU et al., 2006). Possibly the heterogeneous composition of the microbial consortium may have favored the grading of different compounds according to the unique metabolic capabilities of each strain.

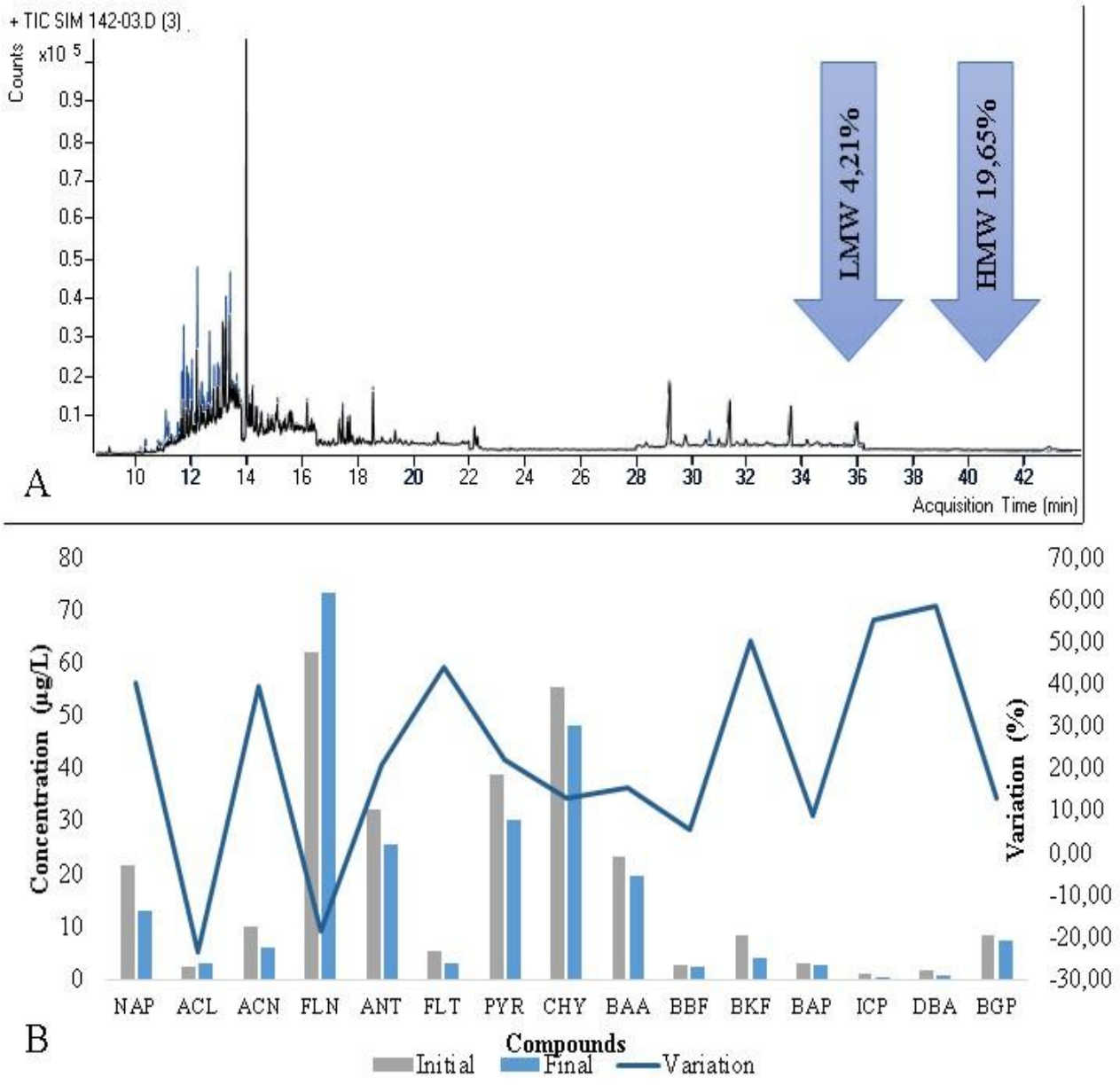
On the other hand, apparently there was resistance in the degradation of PHN, BBF and BAP whose process may have been interfered with due to competitive inhibition, where the more soluble PAHs repress the enzymes used to degrade HMW PAHs (GUPTA; KUMAR; PAL, 2019). In addition, the half-life of 5-ring aromatic hydrocarbons such as BAP can reach a time greater than 200 days (ALEGBELEYE; OPEOLU; JACKSON, 2017), so it would not be possible to observe the decline of these compounds in the experimental period of this work. Boonchan, Britz and Stanley (2000) composed a consortium formed by *Stenotrophomonas maltophilia* and *Penicillium janthinellum* capable of reducing 25 % of BAP in 49 days. In opposition, Machín-Ramírez et al. (2010) reported 65 % depletion of BAP used as single PAH in just 5 days of experiment performed with mixed co-culture of *Penicillium* sp. and *Serratia marcescens*. In general, high values of PAH degradation are reported when the compounds are the only source of carbon and energy, however in contamination events they would rarely be single pollutants or exclusive sources of carbon. As an example, the work by Vaidya et al. (2020), which obtained higher degradation rates of a single PAH (100 %) compared to the

mixture of PYR, CHY, FLT, NAP and PHN (69 %) through the consortium *Pseudomonas* sp., *Burkholderia* sp. and *Mycobacterium* sp. So studies that demonstrate the degradative characteristics of consortia involving mixtures of hydrocarbons or complex sources of carbon such as crude oil can bring relevant information to the process of environmental bio-restoration by multiple microbial systems.

Although the light ΣHPA concentration did not show large variations, compounds such as ACN and NAP had attenuation of 39.64 % and 40.31 %, respectively. NAP is often used as a model PAH to investigate microbial degradation, such as metabolism, genetics and metabolic diversity (MOHAPATRA; PHALE, 2021). As an example, Parab and Phadke, (2020) showed co-biodegradation of naphthalene and phenanthrene in liquid medium from the combination of *Agromyces* sp., *Sphingobacterium* sp., *Chryseobacterium* sp., *Stenotrophomonas* sp, *Pseudomonas* sp. and *Chryseobacterium* sp. reaching a degradation rate of 100 % and 98 %, respectively, in the period of six days. And it is still possible to notice in Figure 40A in blue the increase of more than 18 % and 23 % in the concentration of ACL and FLN, possibly formed from the breakage of other hydrocarbons with a greater number of rings. Despite these findings, Cruz and Marsaioli (2012) suggest that the breakdown of polycyclic aromatic compounds can generate new compounds such as saturated, aromatic, cyclic, acyclic acids and consequently lead to the accumulation of smaller molecules in a given period of time.

Due to the very large difference in the concentration scale of phenanthrene in relation to the other compounds, it was removed from Figure 40B as it was inhibiting the variations between the initial and final time. However, phenanthrene can be seen in Figure 40A with the highest peak.

Figure 40 - Concentration of the 16 priority PAHs in the background and after 7 days of experiment. (A) Chromatographic profile of the 16 priority PAHs. (B) Variation in the concentration of the 16 priority PAHs



NAP=Naphthalene, ACL=Acenaphthylene, ACN=Acenaphthene, FLN=Fluorene, PHN=Phenanthrene, ANT=Anthracene, FLT=Fluoranthene, PYR=Pyrene, CHY=Crysen, BAA=Benzo(a)Anthracene, BBF=Benzo(b) Fluoranthene, BKF=Benzo(K)Fluoranthene, BAP=Benzo(a)Pyrene, ICP=Indene(1,2,3cd)Pyrene, DBA=Dibenzo(ah)Anthracene, BGP=Benzo(ghi)Perylene, LMW=Low Molecular Weight, HMW= High Molecular Weight

Source: Author, 2022.

12.5 CONCLUSION

The results presented here constitute the basis of a protein index of the hydrocarbon-degrading fungi-bacteria consortium that may favor the design of mixed functional consortia with the studied strains.

Differential identification in the presence of crude oil revealed the induction of enzymes related to oxidative stress, transport of molecules, protein synthesis, as well as enzymes involved in the degradation of aromatic compounds, indicating that the cells were responsive to the contaminant. It also presented the identification of a protein related to the production of biosurfactant, suggesting that the consortium can be a source of generation of an attractive byproduct for bioremediation.

Simultaneously, the change in PAH concentrations was obtained following the decreasing order Dibenzo(ah)Anthracene > Indene(1,2,3cd) Pyrene > Benzo(K)Fluoranthene > Fluoranthene > Naphthalene > Acenaphthene > Pyrene > Anthracene > Benzo(a) Anthracene > Chrysene > Benzo(ghi)Perylene > Benzo(a) Pyrene > Benzo(b)Fluoranthene > Phenanthrene.

It is desired to investigate in the future the metabolic cooperation between the microorganisms of the consortium and also their relationship with natural communities in matrices contaminated with petroleum hydrocarbons in order to list the degradation routes and generation of secondary metabolites. That said, understanding the degradation potential of microbial consortia, as well as the enzymes used in the process, lays the foundations for the optimization of a more robust system of environmental recovery.

13 CONSIDERAÇÕES FINAIS

A análise bibliométrica realizada evidenciou a carência de mais estudos relacionados à remediação em sedimento de manguezal contaminado com petróleo principalmente através da utilização de ferramentas das ciências ômicas. Assim a pesquisa sobre dioxigenases pode ser usada como um guia de direcionamento a pesquisas futuras nessa área de conhecimento. Além disso, a recorrente produção de patentes ao redor do mundo nas últimas décadas, enfatizam o tema como alvo de interesse biotecnológico. Lamentavelmente, as patentes registradas no Brasil através do Instituto Nacional de Propriedade Industrial referentes ao processo de tratamento biológico ou enzimático, são de grande maioria de origem internacional com predominância dos Estados Unidos. De modo geral, o patenteamento de processos ou bioprodutos que utilizam enzimas isoladas de células ainda é muito incipiente mesmo sendo comprovada as suas vantagens em relação a células inteiras. Essa pouca representatividade provavelmente seja devido aos altos custos para a investigação e produção enzimática, o que corrobora com o domínio do setor industrial no registro de patentes nessa área. Possivelmente grande parte de estudos acadêmicos com potencialidade para patenteamento não são descobertos ou não possuem financiamento para tal. Assim, acredita-se que com maiores incentivos para interação acadêmico-industrial poderiam potencializar o desenvolvimento tecnológico industrial do país e diminuir os entraves do setor.

A identificação molecular das espécies que compõem o consórcio microbiano revelou a presença de microrganismos pertencentes aos gêneros *Aspergillus, Penicillium*, *Byssochlamys*, *Pseudomonas, Stenotrophomona* e *Bacillus*. Esses gêneros são frequentemente citados na literatura com inúmeras aplicabilidades, dentre elas a biodegradação de compostos aromáticos oriundos de pesticidas, herbicidas, fármacos, corantes têxteis, petróleo e derivados, além da biotransformação de metais traços. A investigação direcionada às espécies de *Allophoma* e *Brevibacillus* é proposta, uma vez que são escassas informações relacionadas a biodegradação de óleo cru e podem abrir portas para obtenção de novos compostos bioativos. E ainda podem ser a chave para compreensão de lacunas da literatura atual. Dessa maneira, as espécies do consórcio revelam indicativos de múltiplas utilidades em processos de biorremediação de sítios contaminados com compostos recalcitrantes.

Notou-se que algumas das espécies do consórcio tais como: *Penicillium* sp.1(R26), *Penicillium* sp.2 (R31) e *Penicillium* sp.1(S40) podem ter características mais expressivas no que diz respeito ao sinergismo microbiano, ponto crucial para a construção de consórcio eficaz. Esses

atributos foram atestados por meio da avaliação do índice de inibição de crescimento, taxa de crescimento, taxa de resistência e classificação do tipo de interação microbiana aliada ao teste de oxidação do 2,6- Diclorofenol Indofenol. No entanto, os testes com as cepas de bactérias (dados não revelados) foram inconsistentes e assim recomenda-se a avaliação conjunta para quantificar a potencialidade de degradação da combinação fungos-bactérias. Assim, a diversidade das espécies e as funções metabólicas das cepas em questão podem fornecer múltiplas aplicabilidades que podem ser otimizadas de acordo com a combinação da composição para suprir a necessidade.

Indícios da produção de manganês peroxidase em fermentação sólida pelos fungos estudados, demonstram a aptidão delas como fonte alternativa para aquisição de enzimas para a redução de poluentes recalcitrantes. Em complementação com esses resultados, os dados de triagem *in sílico* retirados da base Uniprot apontaram a existência putativa de outras enzimas, como por exemplo a lacase, alcano hidroxilase, catecol 1,2 dioxigenase, catecol 2,3 dioxigenase e protocatecol 3,4 dioxigenase envolvidas na degradação de compostos xenobióticos. Nessa ocasião, a ausência de informações mais precisas sobre a cepa do gênero *Allophoma* desperta curiosidade sobre quais mecanismos são utilizados na degradação do petróleo. Isso posto, recomenda-se a investigação mais minuciosa da espécie em questão para entender qual o papel dela em seu processo de decomposição. Por outro lado, a análise proteômica realizada em LC-MS/MS não identificou tais enzimas anteriormente selecionadas. Estima-se que a detecção do conjunto de proteínas tenha ocorrido em função do tempo de incubação em meio de crescimento com óleo, bem como podem estar relacionadas com o esgotamento do oxigênio biodisponível no momento de coleta do experimento. O perfil diferencial das proteínas encontradas em 0 e 7 dias de experimento sugerem uma composição básica de enzimas no período de aclimatação do contaminante e suas alterações. No entanto, estudos futuros com tempos amostrais, tais quais 1, 3, 10 e 15 dias poderiam ratificar as informações encontradas e complementar o entendimento do ciclo de decomposição dos compostos do petróleo, bem como a junção da pesquisa com análises metabólicas. Apesar das enzimas alvo de estudo não terem sido detectadas, outras enzimas com papéis importantíssimos para biocatálise de hidrocarbonetos do petróleo foram identificadas, são elas: Ketol-acid reductoisomerase, S-adenosylmethionine synthase, 4-aminobutyrate--2-oxoglutarate transaminase, Aspartate carbamoyltransferase, Glycine cleavage system H protein, Aconitate hydratase, YceI domain-containing protein, 2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinol hydroxylase e Polyketide cyclase. E ainda destaca-se a possibilidade de produção de

biossurfactante dentre as cepas do consórcio a partir da enzima Beta-ketoacyl-[acyl-carrier-protein] synthase I. Essa informação agrega ainda mais valor às espécies do consórcio, o que pode oferecer componentes valiosos para a confecção de soluções biotecnológicas. Uma vez apontada as principais enzimas relacionadas à degradação de hidrocarbonetos deste consórcio, sugere-se o isolamento das mesmas a fim de aplicá-las de forma mais direcionada ao poluente alvo.

No que diz respeito à degradação dos hidrocarbonetos do petróleo, o consórcio demonstrou indicativos de eficiência em 7 dias de incubação. Essa habilidade pode ser comprovada devido à redução superior a 50 % dos hidrocarbonetos totais do petróleo, atingindo tanto os alcanos leves quanto os pesados. Ou seja, foi possível observar a alteração dos compostos do petróleo desde a fração mais leve a mais complexa, fato que é altamente desejado. Em concomitância verificou-se a redução superior a 40 % dos hidrocarbonetos policíclicos aromáticos prioritários de alto peso molecular, tais como: Benzo(K)Fluoranteno, Indeno(1,2,3cd) Pireno e Dibenzo(ah)Antraceno. Diante desses resultados, acredita-se que o próximo passo seja a otimização da composição do consórcio e o seu o monitoramento metagenômico, a apreciação da biocatálise fora das células e o encapsulamento das enzimas para reduzir alguns empecilhos e consequentemente melhorar a resposta da degradação. Sugere-se a investigação dos metabólitos gerados a partir da deplacção do óleo, bem como avaliar a toxicidade dos compostos resultantes para definir a usabilidade do consórcio na gestão de ambientes contaminados com petróleo ou derivados.

As extraordinárias habilidades biodegradátivas dos microrganismos do consórcio resultam no notável potencial desses para a aplicação em projetos de biorremediação e produção de produtos biotecnológicos. Além da importância no âmbito da pesquisa básica de identificação de espécies e enzimas, este estudo direcionará as atuais estratégias de remediação de ambientes impactados por petróleo ou compostos homólogos para o desenvolvimento de processos mais avançados, possibilitando uma grande variedade de combinações e aplicações dos microrganismos.

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APÊNDICE 1

Consórcio patente n° BR 10 2021 002341 4 depositada no Instituto Nacional da Propriedade Industrial

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

08/02/2021 870210013096 15:41

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Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2021 002341 4

Dados do Depositante (71)

Depositante 1 de 1

Nome ou Razão Social: UNIVERSIDADE FEDERAL DA BAHIA
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PETICIONAMENTO ELETRÔNICO

Esta solicitação foi enviada pelo sistema Peticionamento Eletrônico em 08/02/2021 às 15:41, Petição 870210013096

Continuação - Patente n° BR 10 2021 002341 4 depositada no Instituto Nacional da Propriedade Industrial

Dados do Pedido

Natureza Patente: 10 - Patente de Invenção (PI)

Título da Invenção ou Modelo de Utilidade (54): Consórcio microbiano misto degradador de óleos parafínicos da bacia do Recôncavo-Bahia-Brasil

Resumo: A presente invenção trata de um consórcio microbiano misto (fungos filamentosos e bactérias), isolados do sedimento do manguezal do rio São Paulo da Baia de Todos os Santos- Bahia-Brasil, com aplicação na área de degradação de petróleos parafínicos bruto, mais especificamente petróleos oriundos da bacia do Recôncavo. O produto visa a recuperação de áreas degradadas por atividades petrolíferas. O consórcio compreende 5 (cinco) bactérias Gram negativas e 4 (quatro) Gram positivas; e 24 (vinte e quatro) estirpes de fungos filamentosos. Ele é composto por 8,82 % do gênero Aspergillus, 55,88 % do gênero Penicillium, 5,88 % do gênero Byssochlamys, 2,94% do gênero Allophoma, 8,82% do gênero Pseudomonas, 5,88 % do gênero Bacillus, 8,82 % do gênero tenotrophomonas, 2,94 % do gênero Brevibacillus; os quais constituem bactérias e fungos filamentosos que degradam a compostos saturados, aromáticos e compostos polares (NSO) do petróleo da bacia do Recôncavo-Bahia-Brasil. Esse consórcio pode futuramente ser aplicado diretamente em matrizes contaminadas e/ou ser adaptado e melhorado para aplicação em diversas áreas com problemas ambientais semelhantes.

Figura a publicar: 1

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PETICIONAMENTO ELETRÔNICO Esta solicitação foi enviada pelo sistema Peticionamento Eletrônico em 08/02/2021 às 15:41, Petição 870210013096

APÊNDICE 2

Trabalho apresentado em forma de pôster no XII Seminário Brasileiro de Tecnologia Enzimática (ENZITEC) - 2018

SELECTION OF MICROORGANISMS PRODUCERS OF OXIREDUTASES FOR BIORREMEDIATION APPLICATION

Camila Paim Dantas[1*], Olívia Mª Cordeiro de Oliveira[2], Danusia Ferreira Lima[1],

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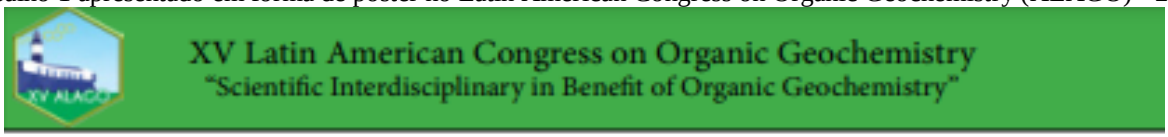
Microorganisms such as bacteria and fungi from marine sources can be used to transform organic compounds, emphasizing the ecological reactions of biocatalysis, biotransformation and biodegradation. The application of enzymes in the transformation of compounds is an efficient way of converting them into more useful substances, since this approach allows the functionalization of the inactive carbon atoms and makes them available in metabolic pathways of the Krebs cycle. The search for new sources of biocatalysts with extraordinary properties becomes interesting and environmentally viable for biotechnological processes linked to the remediation of impacted environments. Thus, the present work aims to track the production of oxirreductase enzymes from isolated strains of microorganisms from coastal environment to the degradation of organic compounds of petroleum. Samples were submitted to qualitative and quantitative assays. The enzymatic production in petri dishes was exposed in categories based on the index of enzymatic activity calculated, subsequently those that presented classification as strongly producing were submitted to submerged and solid state fermentation, where they were quantified to the activities of oxirredutases. The results indicate that the microbial isolates can be used in environmental biocatalysis. The optimization in the production process, as well as the identification and purification of these strains can generate added value to the bioremediation of environments contaminated by oil.

Keywords: Oxygenases, biotransformation, microbial enzymes, PAH-degrader.

APÊNDICE 3

Trabalho 1 apresentado em forma de pôster no Latin American Congress on Organic Geochemistry (ALAGO) - 2018



DEGRADATION OF PARAFFINIC PETROLEUM BY BIORREMEDIATION STRATEGIES IN MANGROVE

CAMILA PAIM DANTAS[A,*], DANUSIA FERREIRA LIMA[A], OLÍVIA MARIA CORDEIRO DE OLIVEIRA[A], ANTÔNIO FERNANDO DE SOUZA QUEIROZ[A]

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Abstract

Biological methods are prominent in the removal of petroleum hydrocarbons. Therefore, this work should aim to investigate the mineralization of crude oil in different bioremediation strategies. The experiment was conducted in a bioreactor prototype with natural attenuation assays; bioaugmentation combined biostimulation with coconut fiber. The results of this study indicate that in this context natural attenuation presented greater oil depletion. This will allow us to gain insight into the applicability of biological strategies to deal with the contamination of oil in sediment.

Keywords: bioaumenation, biostimulation, mangrove sediment.

Introduction

Proper remediation of hydrocarbon impacted environments becomes a difficult task, starting with the choice of an approach to various existing techniques. Biological methods are prominent in the removal of these pollutants, among them are the strategies: natural attenuation, bioaumentation and biostimulation (GARCÍA-SÁNCHEZ et al., 2018).

The intrinsic bioremediation or natural attenuation is a mechanism of natural removal of contaminants, occurring through the autochthonous microbial community capable of biodegrading recalcitrant compounds (DECLERCQ et al., 2012). The bioavailability strategy consists of the addition of strains capable of degrading the target compound, and biostimulation is the incorporation of nutrients into the microorganisms in order to boost degradation (WU et al., 2017).

Commonly, bioremediation is considered a promising method in oil demineralization. However, the efficiency of degradation varies from case to case and may be inconsistent as it is influenced by microbial activity, diversity of hydrocarbon degraders, the ability of inoculants to adapt, and the availability of nutrients to develop (WU et al. al., 2016). Thus, this work should aim to investigate the biodegradability of two different approaches to bioremediation in sediment contaminated by petroleum.

Methods

The mangrove sediment used to simulation It was from Todos os Santos Bay nearby Landulpho Alves de Mataripe renfinery in São Francisco do Conde city, Bahia province, Brazil. According to Lima (2014) this area is without contamination of low, medium and high molecular weight n-alkanes.

The crude oil of Recôncavo Baiano basin and it is composed by 2235,63 mg Kg of pristane, 1570,96 of phytane, 325418,94 mg Kg of total oil hydrocarbons and 72042,57 mg Kg of the unresolved complex mixture.

The consortium used was composed of 38 microbial strains: twenty-six filamentous fungi and twelve bacteria from the Dantas (2016). They formed the consortium encapsulated with coconut fiber according to the methodology of Lima (2014).

The experiment was conducted on a temporary immersion bioreactor prototype described by Dantas (2016). The experimental design was composed of two treatments in triplicate:(NA) sediment artificially contaminated with 1% of oil; (CB) sediment artificially contaminated with 1% of oil and 10 capsules of consortia.

The geochemical monitoring was through of TPH extraction with dichloromethane in a Soxhlet apparatus (IPA, 1996) and analyzed by gas chromatography using the "whole oil" method.

APÊNDICE 4

Trabalho 2 apresentado em forma de pôster no Latin American Congress on Organic Geochemistry (ALAGO) - 2018

SELECTION OF MICROORGANISMS FOR PRODUCTION OF HYDROCARBONOCLASTIC CONSORTIUM

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Abstract

Considering that oil is a pollutant when it is spilled in the environment, it is necessary to use techniques for the treatment of areas contaminated by this oil. Bioremediation is one of the techniques currently used. Thus, this work aimed to isolate and select microorganisms with potential to mineralize the petroleum compounds of the Recôncavo Baiano basin and form a consortium for bioremediation of mangroves. The potential for hydrocarbon degradation of 58 bacterial isolates was tested with 2,6-dichlorophenolindofenol (DCPIP) and 82% of the strains showed a tendency to degrade the saturated fraction, 55% the aromatic fraction and 41% the NSO compounds. It was possible to set up a consortium with 38 potential strains for use in the bioremediation of mangroves contaminated by oil.

Keywords: Coastal areas, bioremediation, dichlorophenolindophenol.

Introduction

Petroleum is a complex mixture of naturally occurring compounds consisting predominantly of hydrocarbons and, to a lesser extent, of organic derivatives (ZÍLIO; PINTO, 2002). Considering that oil is a pollutant that can be propagated in the environment through different routes, it is necessary to develop strategies (CETESB, 2001).

The overall composition of petroleum can be defined by the content of saturated hydrocarbons, aromatics, resins and asphaltenes (ZÍLIO; PINTO, 2002). During the oil extraction and refining stages, accidents occur from leaks in shipping ships or pipelines. According to the database of the Internacional Tanker Owners Pollution Federation Limited (ITOPF), it is estimated that in 2017 there was a spill of 7,000 tons of oil in the Indian Ocean (ITOPF, 2017). Therefore, studies on the use of strategies for these contaminated areas are indispensable with a view to remedy sensitive areas such as coastal areas. According to Trindade (2005), one of the options for remediation techniques is bioremediation, one of the profitable biotechnologies that use autochthonous microorganisms for the decontamination of oceans, coastal areas and soil contaminated by tons of oil dispersed after an incident.

In this way, the objective of this work was to isolate and select microorganisms with the potential to mineralize all petroleum compounds from the Recôncavo Baiano basin, create combination of microbial colonies to be applied as a bioremediation process of mangrove.

Methods

The samples of surface sediment and mangrove leaves were obtained from the Todos os Santos Bay mangrove swamp near the Landulpho Alves refinery in São Francisco do Conde-BA. The petroleum samples were from the Recôncavo Baiano Basin, whose the fractionation was done in vacuum chromatography according to the method of Lima et al. (2012).

The isolation of the bacteria was from the mangrove sediment free from petroleum hydrocarbon contamination, from artificially contaminated sediment by oil for fifteen days and from oil sample. The sediment samples were isolated according to Romeiro (2001) and the oil-only sample, it was made in plates containing nutrient agar and added with 1% oil through surface scattering and incubated for 48 h at 30 ° C. Posteriorly, the fungal strains were isolated and selected by Lima et al. (2017).

The screening of hydrocarbons was carried out by oxidation tests with redox indicator 2,6-dichlorophenol-indophenol (DCPIP) by the method of Hanson et al.

APÊNDICE 5

Trabalho 1 apresentado em forma de slides no Congresso Pesquisa, Ensino e Extensão da UFBA - 2019

Area: BIOTECNOLOGIA

TRABALHO: DOMÍNIO BIOTECNOLÓGICO DE PATENTES RELACIONADAS A REMEDIAÇÃO MICROBIOLÓGICA OU ENZIMÁTICA DEPOSITADAS NO BRASIL

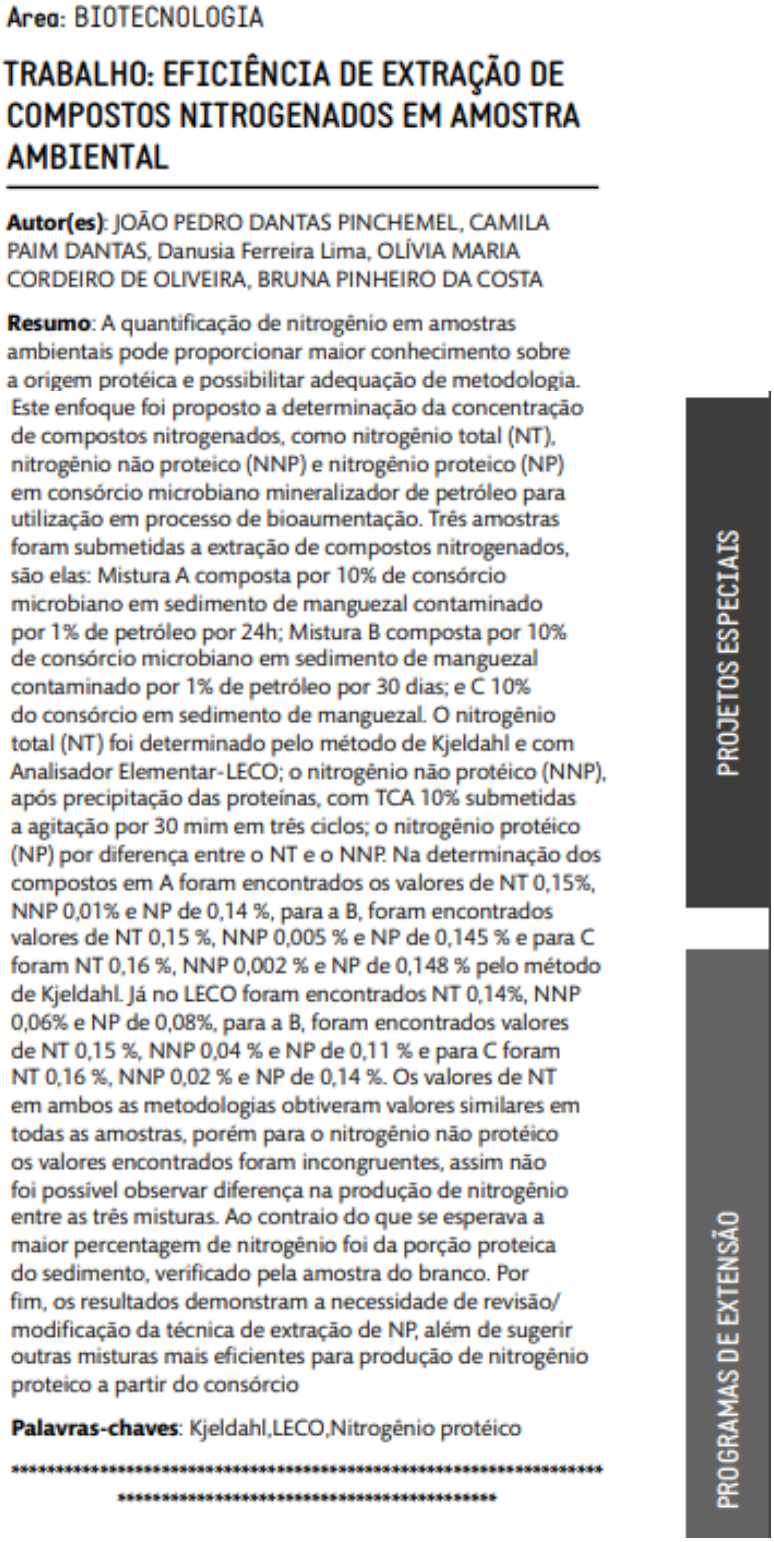
Autor(es): MILENA BARBOSA PIMENTEL, CAMILA PAIM DANTAS, BRUNA PINHEIRO DA COSTA, Danusia Ferreira Lima, OLÍVIA MARIA CORDEIRO DE OLIVEIRA, ICARO THIAGO ANDRADE MOREIRA

Resumo: A ampliação da utilização de microrganismos e/ou enzimas para biotransformações de compostos tornou-se uma tendência nas últimas décadas, visto que podem gerar produtos de alta seletividadeem processos industriais, resulta positivamente em uma diminuição dos efeitos colaterais indesejados eseus subprodutos. O Brasil apresenta elevada competência técnico-cientifica para geraçãode produtos biotecnológicos, entretanto muitos produtos de caráter altamente promissoraainda estão fixados no âmbito acadêmico. Desta forma, objetivou-se nesta prospecção,mapear patentes depositadas no Brasil que detenham tecnologias de remediação desolo/sedimento contaminado usando microrganismos ou enzimas. O delineamentoinvestigativo foi realizado em Maio de 2019, através da pesquisa do código B09C1/10, fazendo uso do sitedo Instituto Nacional de Propriedade Industrial - INPI, focando em processos enzimáticos.Os dados encontrados foram apresentados na forma de gráficos e tabelas elaboradas nosoftware Microsoft Office Excel 2010. A pesquisa resultou em apenas 40 patentesdepositadas entre os anos de 1996 a 2016. Neste cenário foram encontrados depósitos de13 países distribuídos nos continentes americano, europeu, asiático, africano e oceânico. Amaior parte das patentes depositadas nessa temática está sob domínio internacional, assimsendo os EUA destacou-se com 15 % e o Canadá com 12% depósitos de patentes. Essefato pode ser interpretado, principalmente, como competitividade entre esses países e o Brasil. A produçãonacional foi representa por 41% alocados nas regiões Sul, Sudeste e Nordeste. A geraçãode produtos de origem microbiana foi majoritária em relação a utilização direta de enzimasrepresentada por 5 % dos documentos, o que pode demostra a necessidade de investirrecurso para geração de bioprodutos enzimáticos. O Brasil dispõe de um grande potencialno desenvolvimento de tecnologias, porém ainda incipiente para remediação enzimática deambientes contaminados. Esse levantamento ressalta a indispensabilidade do Brasil comoagente produtor inovação tecnológica, utilizando enzimas para recuperação de recursosnaturais.

Palavras-chaves: patente,biotecnologia,biorremediação

Fonte: Caderno de resumos do Congresso Pesquisa, Ensino e Extensão da UFBA. 2019. Salvador :UFBA, p. 679. Disponível em:<https://proext.ufba.br/sites/proext.ufba.br/files/congresso-ufba-2019_caderno-resumos.pdf>

Trabalho 2 apresentado em forma de slides no Congresso Pesquisa, Ensino e Extensão da UFBA - 2019



Area: BIOTECNOLOGIA

TRABALHO: EFICIÊNCIA DE EXTRAÇÃO DE COMPOSTOS NITROGENADOS EM AMOSTRA AMBIENTAL

Autor(es): JOÃO PEDRO DANTAS PINCHEMEL, CAMILA PAIM DANTAS, Danusia Ferreira Lima, OLÍVIA MARIA CORDEIRO DE OLIVEIRA, BRUNA PINHEIRO DA COSTA

Resumo: A quantificação de nitrogênio em amostras ambientais pode proporcionar maior conhecimento sobre a origem protéica e possibilitar adequação de metodologia. Este enfoque foi proposto a determinação da concentração de compostos nitrogenados, como nitrogênio total (NT), nitrogênio não proteico (NNP) e nitrogênio proteico (NP) em consórcio microbiano mineralizador de petróleo para utilização em processo de bioaumentação. Três amostras foram submetidas a extração de compostos nitrogenados, são elas: Mistura A composta por 10% de consórcio microbiano em sedimento de manguezal contaminado por 1% de petróleo por 24h; Mistura B composta por 10% de consórcio microbiano em sedimento de manguezal contaminado por 1% de petróleo por 30 dias; e C 10% do consórcio em sedimento de manguezal. O nitrogênio total (NT) foi determinado pelo método de Kjeldahl e com Analisador Elementar-LECO; o nitrogênio não protéico (NNP), após precipitação das proteínas, com TCA 10% submetidas a agitação por 30 mim em três ciclos; o nitrogênio protéico (NP) por diferença entre o NT e o NNP. Na determinação dos compostos em A foram encontrados os valores de NT 0,15%, NNP 0,01% e NP de 0,14 %, para a B, foram encontrados valores de NT 0,15 %, NNP 0,005 % e NP de 0,145 % e para C foram NT 0,16 %, NNP 0,002 % e NP de 0,148 % pelo método de Kjeldahl. Já no LECO foram encontrados NT 0,14%, NNP 0,06% e NP de 0,08%, para a B, foram encontrados valores de NT 0,15 %, NNP 0,04 % e NP de 0,11 % e para C foram NT 0,16 %, NNP 0,02 % e NP de 0,14 %. Os valores de NT em ambos as metodologias obtiveram valores similares em todas as amostras, porém para o nitrogênio não protéico os valores encontrados foram incongruentes, assim não foi possível observar diferença na produção de nitrogênio entre as três misturas. Ao contraio do que se esperava a maior percentagem de nitrogênio foi da porção proteica do sedimento, verificado pela amostra do branco. Por fim, os resultados demonstram a necessidade de revisão/ modificação da técnica de extração de NP, além de sugerir outras misturas mais eficientes para produção de nitrogênio proteico a partir do consórcio

Palavras-chaves: Kjeldahl,LECO,Nitrogênio protéico

PROJETOS ESPECIAIS

PROGRAMAS DE EXTENSÃO

Fonte: Caderno de resumos do Congresso Pesquisa, Ensino e Extensão da UFBA. 2019. Salvador :UFBA, p. 679. Disponível em:<https://proext.ufba.br/sites/proext.ufba.br/files/congresso-ufba-2019_caderno-resumos.pdf>

APÊNDICE 6

Trabalhos 1 e 2 apresentados em forma de vídeo/pôster no Congresso Virtual UFBA - 2020

Fonte: TV UFBA. Congresso Virtual UFBA. 2020. Disponível em:<https://www.youtube.com/watch?v=i-EGmHEsSwM&t=6s> Acesso em: maio 2020.

Fonte: TV UFBA. Congresso Virtual UFBA. 2020. Disponível em:<https://www.youtube.com/watch?v=rpUiFeH0j40&t=3s> Acesso em: maio 2020.

APÊNDICE 7

Trabalho 1 apresentado em forma de vídeo/pôster no XVI SEBio e I Encontro de estudos Biológicos On-line - 2020

XVI SEBio 2020
I Encontro de
Estudos Biológicos
On-line

Eixo temático: Ciências Moleculares

COMPARAÇÃO DE MÉTODOS DE EXTRAÇÃO DE PROTEÍNAS EM CONSÓRCIO MICROBIANO

DANTAS, Camila Paim*; SANTOS, Cristiane dos; FRANCO, Octávio Luiz; DE OLIVEIRA, Olívia Maria Cordeiro; LIMA, Danusia Ferreira

RESUMO

Introdução: A prospecção de proteínas pode contribuir diretamente na investigação de processos celulares de forma ampla e sistêmica. A identificação de proteínas diferencialmente abundantes pode elucidar informações sobre a biodegradação de hidrocarbonetos de petróleo, e consequentemente revelar fatores importantes ao processo de biorremediação. A base para resultados promissores em análise proteômica é a utilização de um método confiável e reprodutível de extração de proteínas. **Objetivo**: Em vista disso, este trabalho propõe selecionar método de extração de proteínas com maior rendimento frente à composição heterógena de microrganismos num consórcio degradador de petróleo. **Material e métodos**: As cepas do consórcio fúngico (A) fazem parte dos gêneros *Penicilium, Aspergillus, Byssochlamys, Rhodotorula*; o consórcio bacteriano (B) do gênero *Pseudomonas* e *Stenotrophomonas*; e o consórcio misto (C) com a combinação de ambos. Esses foram cultivados em meio líquido Bushnell Haas suplementado com 1% de petróleo ou glicerol a 180 rpm durante 7 dias a 30°C. Foram utilizados dois métodos de extração levando em consideração a estrutura rígida da parede celular dos fungos e, portanto, de difícil acesso ao material proteico. Método I (extração com fenol e precipitação acetato de amônio/metanol), método II (extração com NaCl/ácido cloridrico [0,6 M/0,1%] e precipitação com ácido tricloroacético a 75%). Associado aos diferentes tampões de extração, duas estratégias foram utilizadas para auxiliar na lise celular, uma foi a adição de *beads* de vidros (com 2 ciclos de 5 min de severa agitação alternados com gelo por 1 min). A segunda estratégia foi a submissão do material a 4 ciclos de 2 min de ultrassom (130 W, 20 KHz). As proteínas foram quantificadas com auxílio do Qubit fluorometer (Invitrogen) conforme protocolo do fabricante e visualizadas em gel SDS-PAGE a 12%. **Resultados**: Como resultado, esses métodos produziram diferenças significativas na quantificação de proteínas onde, A, B e C apresentaram 115,0 µg, 183,8 µg e 24 µg, respectivamente para solução com fenol e 12 µg, 10 µg e 2 µg para cloreto de sódio. A visualização de proteínas em gel mostrou maior quantidade de bandas nas amostras extraídas com fenol, corroborando com os dados de quantificação. **Conclusão**: A extração com fenol e precipitação em acetato de amônio/metanol e posterior homogeneização com esferas mostrou-se satisfatória em termos de quantidade e qualidade de material proteico. Assim, nossos resultados fornecem informações úteis para a seleção de métodos de extração adequados para análise proteômica de consórcio de fungos e bactérias.

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Fonte: Dantas, et al. Comparação de métodos de extração de proteínas em consórcio microbiano. Revista Multidisciplinar de Educação e Meio Ambiente. v. 1, n. 2, 2020,: Edição Especial: Anais do I Encontro de Estudos Biológicos On-line. Disponivel em:< https://editoraime.com.br/revistas/index.php/rema/article/view/400/209> Acesso em: out 2020.

APÊNDICE 8

Trabalho 2 apresentado em forma de vídeo/pôster no XVI SEBio e I Encontro de estudos Biológicos On-line - 2020

XVI SEBio 2020
I Encontro de
Estudos Biológicos
On-line

Eixo temático: Microbiologia, Imunologia e Parasitologia.

POTENCIAL ENZIMÁTICO DE FUNGOS FILAMENTOSOS ASSOCIADOS A DEGRADAÇÃO DE PETRÓLEO

PINCHEMEL, João Pedro Dantas*; DANTAS, Camila Paim; DE JESUS, Gisele Maria; OLIVEIRA, Olivia Maria Cordeiro; LIMA, Danúsia Ferreira

RESUMO

Introdução: Hidrocarbonetos presentes no meio ambiente são responsáveis por diversos efeitos biológicos nocivos e apresentam grande potencial de bioacumulação. [1] Fungos, através da atividade de suas enzimas extracelulares, possuem a capacidade de degradação desses compostos, sobretudo os de alto peso molecular. [2] **Objetivo**: O presente estudo visou avaliar a produção das enzimas manganês peroxidase (MnP) e lacase (Lac) pelos fungos filamentosos *Aspergillus sp.*, *Penicilium sp.* 1, *Penicilium sp.* 2, *Penicilium sp.*3 e *Byssochlamys sp.* para geração de bioprotudo. **Material e método**: A determinação da atividade enzimática foi baseada na difusão radial em meio sólido onde, em triplicata, foram cultivados em meio de cultura sabouraud dextrose agar (SDA) e em meio bushnell haas (BH) com petróleo, ambos suplementados com solução de siringaldazina para avaliar a presença de Lac, e do vermelho fenol para a MnP. Dessa forma, os microrganismos foram incubados a 35°C durante 15 dias e o potencial enzimático semi-quantitativo avaliado pela mensuração, em centímetros, do halo de crescimento e halo de descoloração produzido ao redor das colônias a fim de calcular a zona de precipitação. Cálculo correspondente à razão entre o valor médio da zona de crescimento e da zona total de crescimento mais oxidação de cada cepa. **Resultados** Todas as 5 cepas apresentaram resultado positivos para MnP, enquanto para a Lac foi negativo, sugerindo a falta de condições ideais para a produção da enzima. Foi observado que os fungos apresentaram melhor crescimento no meio SDA em comparação ao BH, todavia exibiram melhor produção enzimática em meio BH. O que nos leva a creditar que a maior produção enzimática está relacionada ao agente indutor e não ao crescimento fúngico. O fungo *Byssochlamys sp.* apresentou-se como exceção, demonstrando resultado máximo (9 centímetros) para produção da enzima em ambos os meios de cultura. As cepas apresentaram índices de precipitação mais próximos de 0 no meio mineral, 0.38, 0.31, 0.25, 0.25, 0.41, respectivamente, indicando maior liberação da enzima. **Conclusão:** Os resultados apontam que os isolados de fungos estudados são fontes promissoras de produção da enzima manganês peroxidase, e sua produção pode ser estimulada na presença de hidrocarbonetos presentes no petróleo. Dessa forma, os isolados selecionados podem ser apontados como relevantes para indústria biotecnológica, uma vez que podem gerar um bioproduto potencial agente remediador de áreas afetadas por derramamento de petróleo e seus derivados.

Palavras-chave: biorremediação, hidrocarbonetos, lacase, manganês peroxidase, bioproduto.

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Pinchemel, et al. Potencial enzimático de fungos filamentosos associados a degradação de petróleo. Revista Multidisciplinar de Educação e Meio Ambiente. v. 1, n. 2, 2020,: Edição Especial: Anais do I Encontro de Estudos Biológicos On-line. Disponivel em:<https://editoraime.com.br/revistas/index.php/rema/article/view/508>Acesso em:out 2020.

APÊNDICE 9

Trabalho apresentado em forma de vídeo/pôster no Congresso Brasileiro de Ciências Biológica (COMBRACIB)-2020

POTENCIAL ANTAGÔNICO DE CONSÓRCIO MICROBIANO MISTO DE INTERESSE PARA DEGRADAÇÃO DE ÓLEO PARAFÍNICO

PIMENTEL, Milena Barbosa[1]*; DANTAS, Camila Paim[2]; DE JESUS, Gisele Moraes[3]; OLIVEIRA, Olivia Maria Cordeiro de Oliveira[4]; LIMA, Danusia Ferreira[5]

RESUMO

Introdução: Os microrganismos isolados são importantes promotores da biorremediação de ambientes contaminados por petróleo[1], o consórcio microbiano potencializa a capacidade biodegradadora de óleo. Interações competitivas ocorrem em comunidades microbianas e podem reduzir ou gerar mudanças em suas funções[2]. **Objetivo:** O presente trabalho teve como objetivo avaliar a atividade antagônica e o potencial de biodegradação de óleo por isolados de um consórcio microbiano misto. **Material e métodos**: O teste de antagonismo foi feito pelo método de cultura pareada em placa, respaldados em confronto direto entre as diferentes cepas de fungo e bactéria, o período de incubação foi de quinze dias para fungos e sete para bactérias a 30 °C. Os resultados foram determinados pelo índice de inibição de crescimento dos antagônicos e coloração apresentada pelo meio de cultura. Foram isolados sete representantes de fungos filamentosos: *Penicillium* sp.3, *Penicillium* sp.4, *Aspergillus* sp.1 *e Byssochlamys* sp1. Cinco representantes de bactérias, sendo dos gêneros *Pseudomonas* sp.1, *Pseudomonas* sp.2, *Bacillus* sp. *e Stenotrophomonas* sp. Todos degradadores de diferentes frações do petróleo. **Resultados**: Os resultados mostraram que o fungo *Aspergillus* sp.1 inibiu o crescimento de seis fungos e de todas as bactérias confrontadas, aparentemente houve produção de metabólito extracelular secundário ao confrontar o gênero *Byssochlamys* sp1. Os gêneros *Penicillium* sp.3 e *Byssochlamys* sp1 revelaram as maiores atividades degradadoras. A bactéria *Pseudomonas* sp.2 exibiu maior crescimento em relação aos outros gêneros bacterianos. Representantes inibidos apresentaram redução na biodegradação. **Conclusão**: Dessa forma, os resultados obtidos pelo teste de atividade antagônica fornecem informações relevantes para a escolha de microrganismos e criar uma composição mais eficiente de consórcio misto, buscando obter-se o melhor desempenho na degradação de óleo parafínico em processo de biorremediação de ambientes impactados pelo petróleo ou seus derivados.

Palavras-chave: antagonismo; biorremediação; consórcio; petróleo.

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https://conbracib.com.br

Pimentel, et al. Potencial antagônico de consórcio microbiano misto de interesse para degradação de óleo parafínico. Revista Multidisciplinar De Educação E Meio Ambiente, v.1, n. 1, 150., 2020. Disponível em:< https://editoraime.com.br/revistas/index.php/rema/article/view/290>Acesso em:2020.

APÊNDICE 10

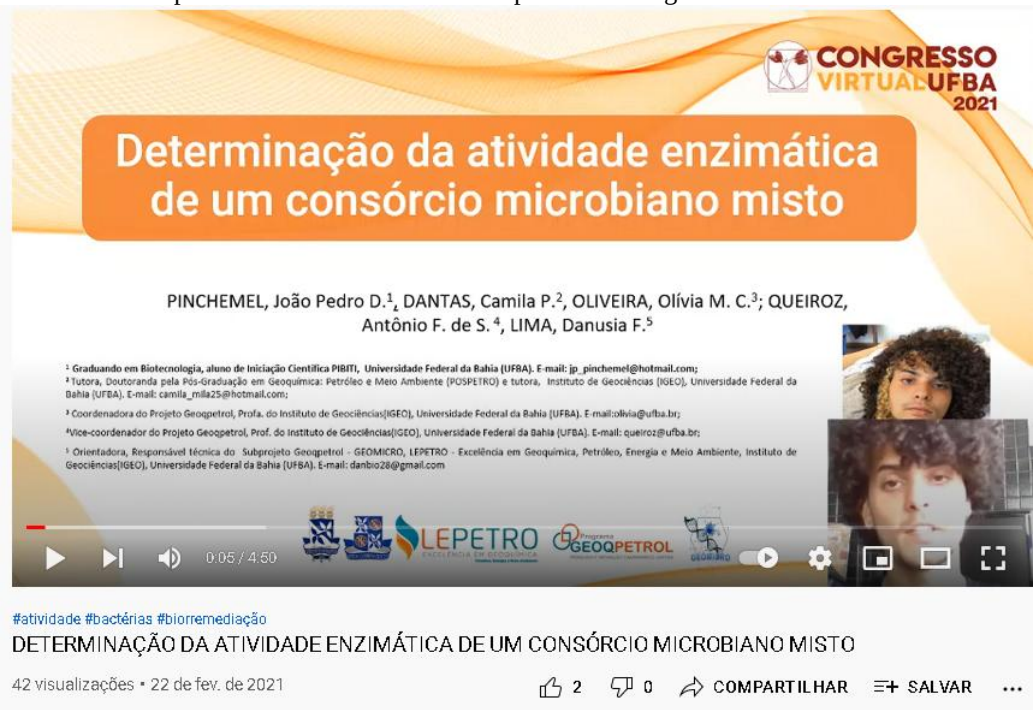
Trabalhos 1 e 2 apresentados em forma de vídeo/pôster no Congresso Virtual UFBA - 2021

Fonte: TV UFBA. Congresso Virtual UFBA. 2021. Disponível em:<https://www.youtube.com/watch?v=c8RSMaDsdPk&t=2513s> Acesso em: fev 2021.

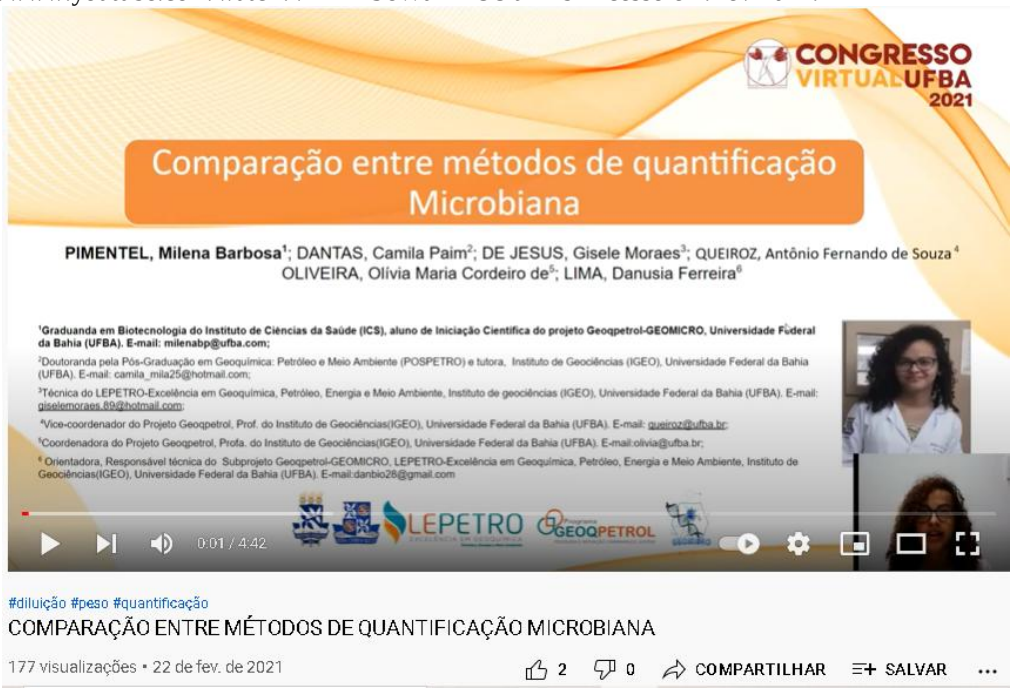
Fonte: TV UFBA. Congresso Virtual UFBA. Disponível em:<https://www.youtube.com/watch?v=ZKjcO502n7s>Acesso em: fev 2021.

APÊNDICE 11

Trabalhos 3 e 4 apresentados em forma de vídeo/pôster no Congresso Virtual UFBA - 2021



Fonte: TV UFBA. Congresso Virtual UFBA. 2021. Disponível em:<https://www.youtube.com/watch?v=ZKZ9eWa7Z-U&t=24s>Acesso em: fev 2021.



Fonte: TV UFBA. Congresso Virtual UFBA. 2021. Disponível em:<https://www.youtube.com/watch?v=YfP3YS8pxCc&t=58s>Acesso em: fev 2021.

APÊNDICE 12

Trabalho 1 apresentado em forma de vídeo/pôster no Congresso UFBA 75 anos em 2021. Vídeo disponível em:<https://www.youtube.com/watch?v=TDvmflnHaEs>

Área: CIÊNCIAS EXATAS E DA TERRA – GEOCIÊNCIAS

TRABALHO: MIX MICROBIANO AUTÓCTONE DE MANGUEZAL NA DEGRADAÇÃO DE HIDROCARBONETOS

Autor(es): CAMILA PAIM DANTAS, OLÍVIA MARIA CORDEIRO DE OLIVEIRA, DANUSIA LIMA, ANTONIO FERNANDO DE QUEIROZ

Resumo: Os manguezais sustentam comunidades microbianas ricas em propriedades uteis a indústria biotecnológica. A dinâmica variável desses ambientes proporciona diferentes mecanismos a algas, fungos e bactérias são capazes de promover a degradação de compostos orgânicos, tal qual o petróleo. Uma abordagem mista de microbianos pode agir em complementaridade de função entre as espécies e impulsionar a transformação desses compostos de forma eficaz e sustentável com maior potencial de degradação quando comparada às abordagens de monocultura. Dessa forma, esse trabalho deve como objetivo avaliar a degradação de hidrocarbonetos do petróleo a partir de um consórcio microbiano misto para futuras aplicações em processos de remediação de manguezais contaminados. Os resultados demonstraram que a mineralização do petróleo pelo consórcio foi superior a atenuação natural sem a presença dos microrganismos, atingindo valores acima de 50%. Assim sendo, é possível indicar a utilização desses em processos de degradação de hidrocarbonetos do petróleo e compostos homólogos indesejáveis.

Palavras-chave: Biodegradação, Biorremediação, Consórcio microbiano, Petróleo

Fonte:Bertissolo, F.Caderno de resumosdo Congresso UFBA 75 anos, Salvador:UFBA, 2021, p. 685. Disponível em:< https://congresso75anos.ufba.br/wp-content/uploads/2021/11/congresso-ufba-75anos_caderno-resumos.pdf>

Trabalho 2 apresentado em forma de vídeo/pôster no Congresso UFBA 75 anos em 2021. Vídeo disponível em <https://www.youtube.com/watch?v=fP9V03AtCE4&t=1s>

Área: BIOTECNOLOGIA

TRABALHO: DIVERSIDADE FUNCIONAL NA ANÁLISE PROTEOGENÔMICA DE CONSÓRCIO MICROBIANO DE MANGUEZAL

Autor(es): JOÃO PEDRO DANTAS PINCHEMEL, CAMILA PAIM DANTAS, DANUSIA LIMA, OLÍVIA MARIA CORDEIRO DE OLIVEIRA, ANTONIO FERNANDO DE QUEIROZ, EDDY JOSÉ FRANCISCO DE OLIVEIRA

Resumo: O presente trabalho visou a avaliação in silico da presença das enzimas lacase, manganês peroxidase, lignina peroxidase, alcano hidroxilase, catecol 1,2 dioxigenase, catecol 2,3 dioxigenase e protocatecol 3,4 dioxigenase a partir da comparação de sequências de DNA de um consórcio microbiano de interesse biotecnológico. O estudo foi realizado através das plataformas UNIPROT e NCBI, utilizando como referência as sequências de identificação de fungos e bactérias presentes no consórcio misto. Na base de dados UNIPROT, foram selecionadas espécies identificadas como produtoras destas enzimas, no NCBI, a similaridade dessas espécies foi comparada com organismos presentes no consórcio e as semelhanças entre os grupos de proteínas foram plotados no diagrama de Venn. Ao total, 1159 proteínas associadas à biorremediação foram encontradas envolvidas com o consórcio . Dentre as cepas, destacam-se a Bacillus sp. 1, Penicillium sp. 2 e Pseudomonas sp. 2, com 6,7%, 6,5% e 5,5%, respectivamente, de similaridade entre as proteínas. O táxon que apresentou maior número de enzimas foi o Bacillus sp.1 com 43 enzimas Lacase, 1 enzima Alcano hidroxilase, 2 enzimas Catecol 1,2 dioxigenase, 31 enzimas Catecol 2,3 dioxigenase e 1 enzima Protocatecol 3,4 dioxigenase. Por meio do diagrama de Venn, verificou-se que 72,7% das cepas apresentaram grupos similares de enzimas, 24,2% estão presentes a catecol 1,2 dioxigenase e apenas 1% é compartilhada entre alcano hidroxilase, catecol 2,3 dioxigenase e protocatecol 3,4 dioxigenase. A metanálise de enzimas reflete as funções da microbiota na biorremediação e o alto intercâmbio metabólico ocorrente. Todavia, nem sempre é possível correlacionar a população microbiana com a população de proteínas, pois diferentes espécies de microrganismos expressam diferentes proteínas sob condições ambientais variáveis. Dessa forma, a análise in silico descreve as principais funções e possíveis localizações das proteínas em uma determinada espécie.

Palavras-chave: biorremediação, enzimas microbianas, biodegradação enzimática, dioxigenases

Fonte:Bertissolo, F.Caderno de resumosdo Congresso UFBA 75 anos, Salvador:UFBA, 2021, p. 685. Disponível em:< https://congresso75anos.ufba.br/wp-content/uploads/2021/11/congresso-ufba-75anos_caderno-resumos.pdf>

APÊNDICE 13

Artigo derivado da tese publicado na revista Anais da Academia Brasileira de Ciências em 2021

An Acad Bras Cienc (2021) 93(Suppl. 3): e20210296 DOI 10.1590/0001-3765202120210296
Anais da Academia Brasileira de Ciências | Annals of the Brazilian Academy of Sciences
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www.scielo.br/aabc | www.fb.com/aabcjournal

MICROBIOLOGY

Bioprospection of ligninolytic enzymes from marine origin filamentous fungi

CAMILA P. DANTAS, JOÃO PEDRO D. PINCHEMEL, GISELE M. DE JESUS, MILENA B. PIMENTEL, OLÍVIA MARIA C. OLIVEIRA, ANTÔNIO FERNANDO S. QUEIROZ & DANUSIA F. LIMA

Abstract: Fungi are excellent producers of extracellular enzymes. Therefore, the present study aimed to investigate the screening of marine fungi, which are laccase and manganese peroxidase potential producers, in solid fermentation for future applications in bioremediation processes of contaminated sites. For this purpose, two-level factorial planning was adopted, using time (6 and 15 days) and the absence or presence of oil (0 and 1%) as factors. The semi-quantitative evaluation was carried out by calculating radial growth, enzyme activity and enzyme index by measuring phenol red or syringaldazine oxidation halo. The results showed that all the studied strains showed a positive result for manganese peroxidase production, with an enzymatic activity in solid medium less than 0.61, indicating a strongly positive activity. Through the enzyme index, the study also showed prominence for *Penicillium* sp. strains, with values > 2. The enzyme index increase in oil presence and the inexpressive use of the genera studied for ligninolytic enzymes production from crude oil demonstrated these data importance for fermentative processes optimization. Considering the ability of these strains to develop into recalcitrant compounds and the potential for manganese peroxidase production, they are indicated for exploitation in various bioremediation technologies, as well as other biotechnological applications.

Key words: bioremediation, enzymatic activity, manganese peroxidase, mycoremediation.

INTRODUCTION

The production of enzymes from microorganisms exposes great biotechnological, industrial and environmental interest due to its wide catalytic activities variety, large-scale production possibility, adaptation ease, renewability capacity and propensity for socioeconomic and environmental merits (Behbudi et al. 2021). Therefore, ligninolytic enzymes have a background for white biotechnology, with great potential for generating bio-products with high added value from natural biomass, following the green agenda (Bilal & Iqbal 2020).

Ligninolytic enzymes (LEs) are made up of three main representatives: lignin peroxidase (LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13) and laccase (Lac, EC 1.10.3.2), along with others accessory enzymes (Wong 2009) and have the function of catalyzing modifications or degrading lignin into less complex molecules (Asemoloye et al. 2020). These enzymes have great versatility that covers a wide spectrum of use in bioremediation, and it is pointed out its complex recalcitrant compounds degradation and mineralization capacity, such as petroleum compounds and their derivatives (Asemoloye et al. 2020), pesticides (Zeng et al. 2017), drugs, such as endocrine disrupters (Eldridge et al. 2017)

Fonte: DANTAS, Camila P. et al. Bioprospection of ligninolytic enzymes from marine origin filamentous fungi. Anais da Academia Brasileira de Ciências, v. 93, 2021.Disponivel em:<https://doi.org/10.1590/0001-3765202120210296>Acesso em: jan 2022.

APÊNDICE 14

Quadro 9 - Identificação molecular e quantificação dos microrganismos do consórcio em câmara de Newbauer

Tipo	Código	Número de acesso (NCBI)	Espécie mais próxima	Viabilidade (%)	Abs 492nm	Abs 600nm	UFC/mL (x10 ^{6})
BACTÉRIAS	OH4	MW881204	*Bacillus sp.*	84,38	0,692	0,576	16,2
	PD4	NI*	*NI**	89,36	0,656	0,587	16,8
	PD5	MW881200	*Stenotrophomonas sp.*	64,58	0,538	0,439	6,2
	PD6	MW881201	*Pseudomonas sp.*	60,92	0,813	0,628	21,2
	PD7	MW881202	*Pseudomonas sp.*	71,30	0,066	0,04	15,4
	PD8	MW881203	*Stenotrophomonas sp.*	72,81	0,964	0,772	66,4
	RA2	MW881196	*Pseudomonas sp.*	83,16	0,254	0,194	32,6
	RB4	MW881197	*Bacillus sp.*	72,43	1,007	0,806	6,7
	RC5	MW881198	*Brevibacillus sp.*	100,00	0,262	0,198	39,6
	RC6	MW881199	*Stenotrophomonas sp.*	85,71	0,502	0,39	3,6
FUNGOS	A79	MW865722	*Penicillium sp.*	97,58	1,756	1,653	24,2
	A80	MW865723	*Penicilium sp.*	41,37	3,405	3,191	30,2
	A83	MW865724	*Penicillium sp.*	27,30	3,741	3,469	21,4
	A84	MW865725	*Penicillium sp.*	33,18	2,768	2,560	14,0
	N101	MW865728	*Byssochlamys sp.*	100,00	2,084	1,719	69,8
	N102	MW865729	*Penicillium sp.*	7,45	2,244	1,841	4,2
	N82	MW865726	*Penicillium sp.*	NC**	NC**	NC**	NC**
	N89	MW855903	*Byssochlamys sp.*	85,37	1,115	0,997	7,0
	N96	MW865727	*Penicillium sp.*	43,65	2,791	2,550	44,0
	R2	MW865711	*Aspergillus sp.*	NC**	NC**	NC**	NC**
	R11	MW855898	*Penicilium sp.*	93,45	1,984	1,879	42,8
	R16	MW855899	*Aspergillus sp.*	3,88	3,877	3,649	1,0
	R26	MW855900	*Penicilium sp.*	38,33	1,599	1,470	4,6
	R27	MW865712	*Penicilium sp.*	32,36	3,331	3,127	20,0
	R28	MW865713	*Penicilium sp.*	82,50	1,354	1,288	13,2
	R30	MW865714	*Penicilium sp.*	22,03	2,315	2,152	26,0
	R31	MW855901	*Penicillium sp.*	56,22	2,812	2,573	26,2
	R33	MW865715	*Aspergillus sp.*	NC**	NC**	NC**	NC**
	S36	MW865716	*Penicilium sp.*	52,23	2,598	2,440	16,4
	S38	MW865717	*Penicilium sp.*	98,58	2,061	1,911	97,0
	S39	MW865718	*Penicilium sp.*	43,75	1,707	1,572	11,2
	S40	MW855902	*Penicilium sp.*	85,71	1,017	0,925	3,6
	S41	MW865719	*Penicilium sp.*	23,41	2,885	2,688	36,8
	S45	MW865720	*Allophoma sp.*	72,82	1,346	1,226	15,0
	S52	MW865721	*Penicilium sp.*	88,30	1,680	1,578	33,2

NI* Não identificado, NC* Não contabilizado. Fonte: Autor, 2022.

APÊNDICE 15

Quadro 10 - Resumo das principais enzimas envolvidas na degradação de compostos do petróleo e análogos xenobióticos

Código	Enzima	Composto degradado
(EC 1.1.1.303)	Acetoin dehydrogenase	Acetoina ou butanodiol.
(EC 1.14.15.3)	Alkane monooxygenase	Heptano, n-hexadecano, óleo diesel, n-alcanos de cadeia média e longa (C12-C30), tetradecano, n-octadecano, n-eicosano, pirrolidinas, pirrolidinonas, azetidinas, azetidinonas, piperidinas e piperidinonas.
(EC 1.1.1.1)	Alcohol dehydrogenase	Propanol, naftaleno, ácidos gordos, (S) -propano-1,2-diol, biossíntese de 3-metilbutanol, acetileno, etanol I, etanol II, L-isoleucina II, L-leucina III, L-metionina III, L-fenilalanina III, L-triptofano V, L-tirosina III, L-valina II, noradrenalina, adrenalina e degradação de fitol serotonina.
(EC 1.11.2.4)	Alpha-fatty acid hydroxylase	*n*-dodecano, *n*-hexadecano, *n*-octadecano, *n*-docosano,, n-tetracosano, pristano, tolueno, fenol e naftaleno.
(EC 1.1.1.9)	Aryl-alcohol dehydrogenase	fenilalanina, tolueno, tirosina, xileno, 1,3-dimetilbenzeno em 3-metilbenzoato, 1,4-dimetilbenzeno em 4-metilbenzoato , 2,5-xilenol e 3,5-xilenol, 3-clorotolueno II, m-cresol, salicina e tolueno em benzoato.
(EC 1.13.11.-)	Aromatic ring-opening dioxygenase	Compostos aromáticos
(EC1.14.14.92)	Benzoate 4- monooxygenase	Aminobenzoato e benzoato.
(EC 1.2.1.28)	Benzaldehyde dehydrogenase (NAD+)	Tolueno, xileno, aminobenzoat, 1,3-dimetilbenzeno em 3-metilbenzoato, 1,4-dimetilbenzeno em 4-metilbenzoato , 3-clorotolueno II , benzoato II, benzoato III, D-fenilglicina , mandelato I , salicina e salicortina.
(EC 2.7.2.7)	Butyrate kinase	(S) -propano-1,2-diol, L-treonina I e fermentação de piruvato em butanoato.
(EC1.14.13.230)	Butane monooxygenase	Alcanos C3-C6 lineares e alifáticos ramificados, butano.
(EC 1.13.11.1)	Catechol 1,2-dioxygenase	3-clorocatecol, fenol, benzoato, clorociclohexano, clorobenzeno, fluorobenzoato e tolueno, catecol em beta-cetoadipato.
(EC 1.13.11.2)	Catechol 2,3-dioxygenase	Fenol, benzoato, clorociclohexano e clorobenzeno, estireno, xileno, 2-nitrotolueno, catecol em 2-hidroxipentadienoato I, catecol em 2-hidroxipentadienoato II, tolueno em 2-hidroxipentadienoato, hidroxipentadienoato e tolueno em 2-hidroxipentadienoato I.
(EC 1.14.14.25)	Cytochrome P450 hydroxylase	Benzo [a] pireno
	Dihydroxycyclohexadienecarboxylate dehydrogenase	p-xileno.
(EC1.14.13.131)	Dimethylsulfide monooxygenase	Dimetil sulfeto I.
(EC 1.18.1.3)	Ferredoxin-NAD+ reductase	Degradação de ácidos graxos e fosforilação oxidativa.
(EC 1.7.1.17)	FMN-dependent NADH-azoreductase	Compostos aromáticos azo.

Fonte: Elaborado a partir do Uniprot, KEGG ENZYME e Brenda,2022.

Quadro 10. Resumo das principais enzimas envolvidas na degradação de compostos do petróleo e análogos xenobióticos (continuação)

Código	Enzima	Composto degradado
(EC 1.2.1.46)	Formaldehyde dehydrogenase	Cloroalcano e cloroalceno, formaldeído IV e oxidação de metanol a dióxido de carbono.
(EC 1.17.1.9)	Formate dehydrogenase	Acetil coenzima A, glioxilato e dicarboxilato, metano, formato em CO2, oxalato III, degradação de oxalato VI, nucleobases de purina I e nucleobases de purina II.
(EC 1.11.1.14)	Lignina peroxidase	Lignina, álcool veratrílico, n-propanol, (1,2-dihidroxibenzeno), 4-clorocatecol (4-CC), 4,5-diclorocatecol (4,5-DCC) e 4-metilcatecol (4-MC), Corante GLL azul direto, xileno cianol, fucsina, rodamina B, azul de metileno, verde malaquita, laranja de metila e outros azos sulfonados.
(EC 1.10.3.2)	Laccase	Biossíntese de gossipol, justicidina B, matairesinol e sesamina.
(EC 1.11.1.13)	Manganese peroxidase	Manganês e componentes fenólicos da lignina, pentaclorofenol, dioxinas, tetraciclina, oxitetraciclina e aflatoxina B1.
(EC 1.14.18.3) (EC 1.14.13.25)	Methane monooxygenase	Alcanos cíclicos, alcenos e metano a metanol I, alcano de cadeia curta (C 2 a C 4).
(EC 1.1.1.244)	Methanol dehydrogenase	Metanol em dióxido de carbono e metanol em formaldeído II.
(EC 1.14.13.7)	Phenol hydroxylase	Fenol e compostos aromáticos.
(EC 1.13.11.3)	Protocatchetuate 3,4-dioxygenase	4-hidroxmandelato, hidrocarboneto policíclico aromático, galato, benzoato, 4-sulfocatecol e protocatecato II.
(EC 1.18.1.1)	Rubredoxin-NAD+ reductase	Octanas e ácidos graxos.
(EC 1.14.13.1)	Salicylate 1-monooxygenase	Fenol, naftaleno, HPA's, dioxinas, clorossalicilato e metilsalicilato, salicilato I.
(EC 1.11.1.16)	Versatile peroxidases	Hidrocarbonetos aromáticos e lignina.
(EC 1.13.11.41)	2, 4 dihydroxyacetophenonedioxygenase	Naftaleno, bisfenol
(EC 5.3.2.8)	4-oxalomesaconate tautomerase	Tolueno, o-xileno, 3-etiltolueno e 1,2,4-trimetilbenzeno, galato I, galato II, metilgalato, protocatecuato I (via de meta-clivagem), seringado e benzoato.
(EC 1.13.11.39)	Biphenyl-2,3-diol 1,2-dioxygenase	Bifenil e gama-hexaclorociclohexano
(EC 5.5.1.7)	Chloromuconate isomerase	HPA, 1,4-diclorobenzeno
(EC 3.1.1.45)	Carboxymethylenebutenolidase	Tolueno
(EC 1.2.1.10)	Acetaldehyde dehydrogenase	HPA, benzoato, dioxina, xileno, fenóis, metilfenóis e catecóis
(EC 4.1.3.39)	4-hydroxy-2-oxovalerate aldolase 1	HPA, benzoato, dioxina, xileno,
(EC 1.3.1.40)	2-Hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase	Catabolismo de hidrocarbonetos aromáticos
(EC 1.14.12.18)	Biphenyl 2,3-dioxygenase	Naftaleno
(EC 1.3.1.29)	2-Dihydroxy-1,2-dihydronaphthalene dehydrogenase	Hidrocarbonetos aromáticos bicíclicos
(EC 1.14.12.12)	Naphthalene 1,2-dioxygenase	Naftaleno

Fonte: Elaborado a partir do Uniprot, KEGG ENZYME e Brenda,2022.

APÊNDICE 16

Quadro 11 - Concentração dos *n*-alcanos (nC_8 a nC_{40}) do petróleo da Bacia do Recôncavo Baiano(Maringa 1, P-106)

***n*-Alcano**	**Concentração**		**LQM* (mg/L)**
	mg/L	**µg/L**	
n-C_8	74,03	74032,00	4,16
n-C_9	138,39	138390,00	4,17
n-C_{10}	171,39	171389,00	5,08
n-C_{11}	211,05	211051,00	5,32
n-C_{12}	206,50	206495,00	4,46
n-C_{13}	210,82	210817,00	4,03
n-C_{14}	217,62	217621,00	3,56
n-C_{15}	208,98	208983,00	2,57
n-C_{16}	207,43	207434,00	2,26
n-C_{17}	213,72	213722,00	2,34
n-C_{18}	194,45	194454,00	2,76
n-C_{19}	213,85	213850,00	3,07
n-C_{20}	191,94	191940,00	3,43
n-C_{21}	195,97	195972,00	3,67
n-C_{22}	196,60	196603,00	3,74
n-C_{23}	210,04	210040,00	3,70
n-C_{24}	189,88	189881,00	3,53
n-C_{25}	208,00	208000,00	3,24
n-C_{26}	188,06	188058,00	2,95
n-C_{27}	205,56	205557,00	2,71
n-C_{28}	169,83	169828,00	2,58
n-C_{29}	168,82	168818,00	2,65
n-C_{30}	119,98	119979,00	2,66
n-C_{31}	108,92	108918,00	3,55
n-C_{32}	79,54	79540,00	2,99
n-C_{33}	67,99	67992,00	2,65
n-C_{34}	53,14	53144,00	2,54
n-C_{35}	41,55	41545,00	2,96
n-C_{36}	28,22	28224,00	2,82
n-C_{37}	28,65	28649,00	3,30
n-C_{38}	20,90	20899,00	2,40
n-C_{39}	20,99	20989,00	3,81
n-C_{40}	16,47	16470,00	4,02

* <LQM: Limite de Quantificação.
Fonte: Autor, 2022.

Quadro 12 - Valores dos alcanos e parâmetros geoquímicos da amostra de petróleo da Bacia do Recôncavo Baiano

Alcano ou Parâmetro	**Concentração**		**LQM ($mg.L^{-1}$)**
	$mg.L^{-1}$	**$\mu g.L^{-1}$**	
Pristano	74,68	74678,00	2,29
Fitano	45,49	45490,00	2,90
HTP	7354,58	7354581,00	N.A.
UCM	956,77	956769,00	N.A.
Pristano/Fitano	1,64		N.A.
Pristano/n-C_{17}	0,35		N.A.
Fitano/n-C_{18}	0,23		N.A.
HTP/UCM	7,68		N.A.

* HTP: Hidrocarbonetos Totais do Petróleo, UCM: *Unresolved Complex Misture*, Misturas Complexas não Resolvidas, N.A.: Não se aplica.
Fonte: Autor, 2022.

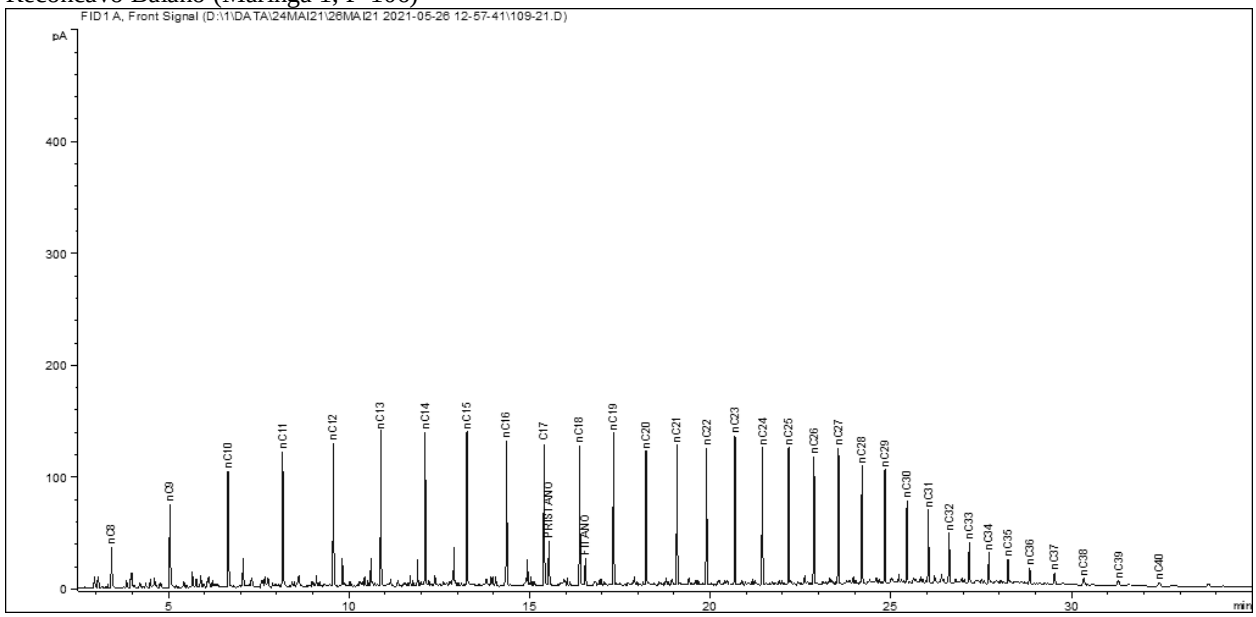
Quadro 13 - Valores dos hidrocarbonetos policíclicos aromáticos prioritários da amostra de petróleo da Bacia do Recôncavo Baiano (Maringa 1, P-106)

Composto	**Concentração**	
	$mg.L^{-1}$	**($\mu g.L^{-1}$)**
Naftaleno	0,00639	6,39
Acenaftileno	0,01130	11,30
Acenafteno	0,01422	14,22
Fluoreno	0,09188	91,88
Fenantreno	0,35846	358,46
Antraceno	0,02571	25,71
Fluoranteno	0,00071	0,71
Pireno	0,03118	31,18
Criseno	0,04120	41,20
Benzo(a)Antraceno	0,01404	14,04
Benzo(b)Fluoranteno	0,00091	0,91
Benzo(K)Fluoranteno	0,00866	8,66
Benzo(a) Pireno	0,00224	2,24
Indeno(1,2,3cd)Pireno	0,00081	0,81
Dibenzo(ah)Antraceno	0,00044	0,44
Benzo(ghi)Perileno	0,00235	2,35

Fonte: Autor, 2022.

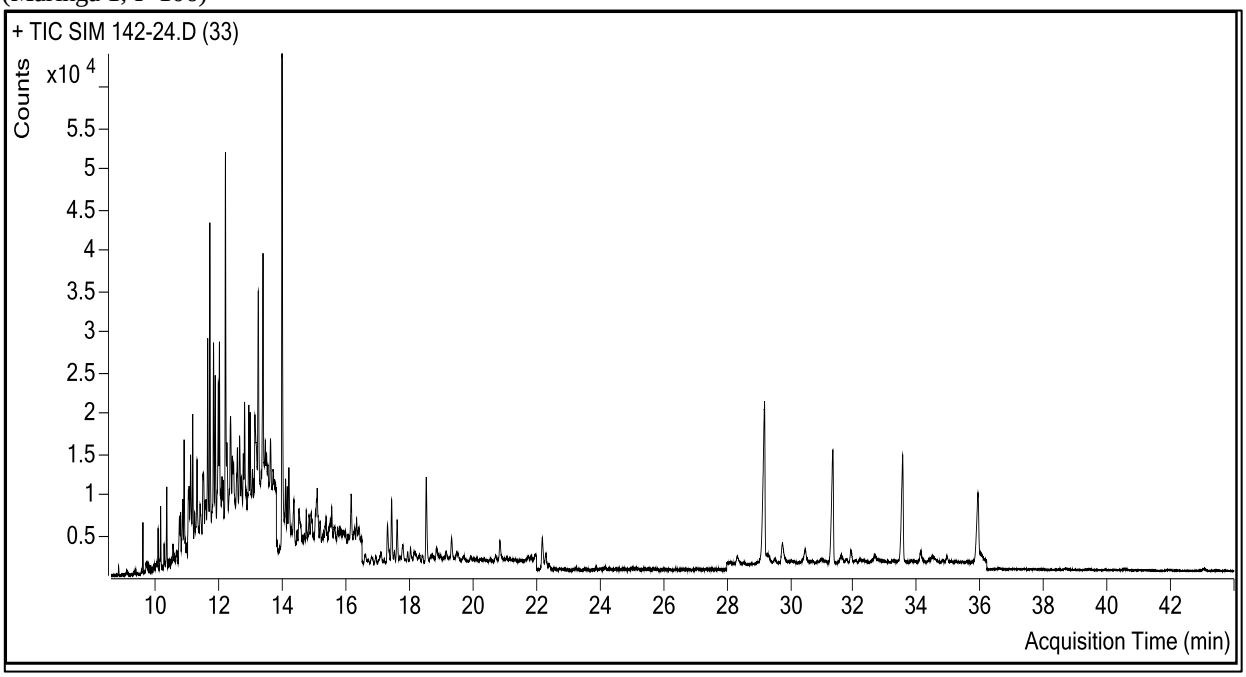
APÊNDICE 17

Figura 41 - Perfil cromatográfico dos *n*-alcanos (nC_8 a nC_{40}) e isoprenoides da amostra de petróleo da Bacia do Recôncavo Baiano (Maringa 1, P-106)



Fonte: Autor, 2022.

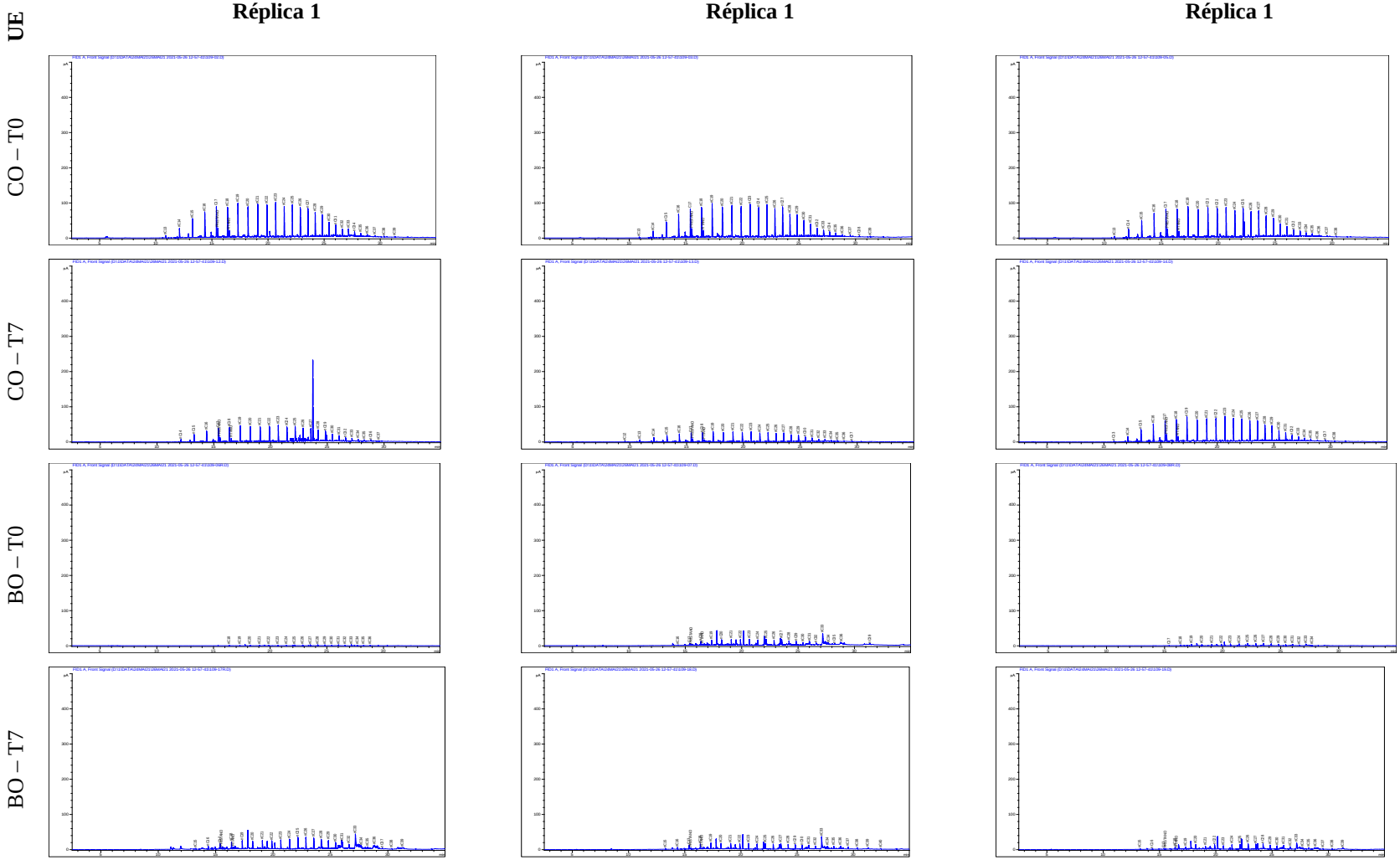
Figura 42 - Perfil cromatográfico dos HPAs prioritários da amostra de petróleo da Bacia do Recôncavo Baiano (Maringa 1, P-106)



Fonte: Autor, 2022.

APÊNDICE 18

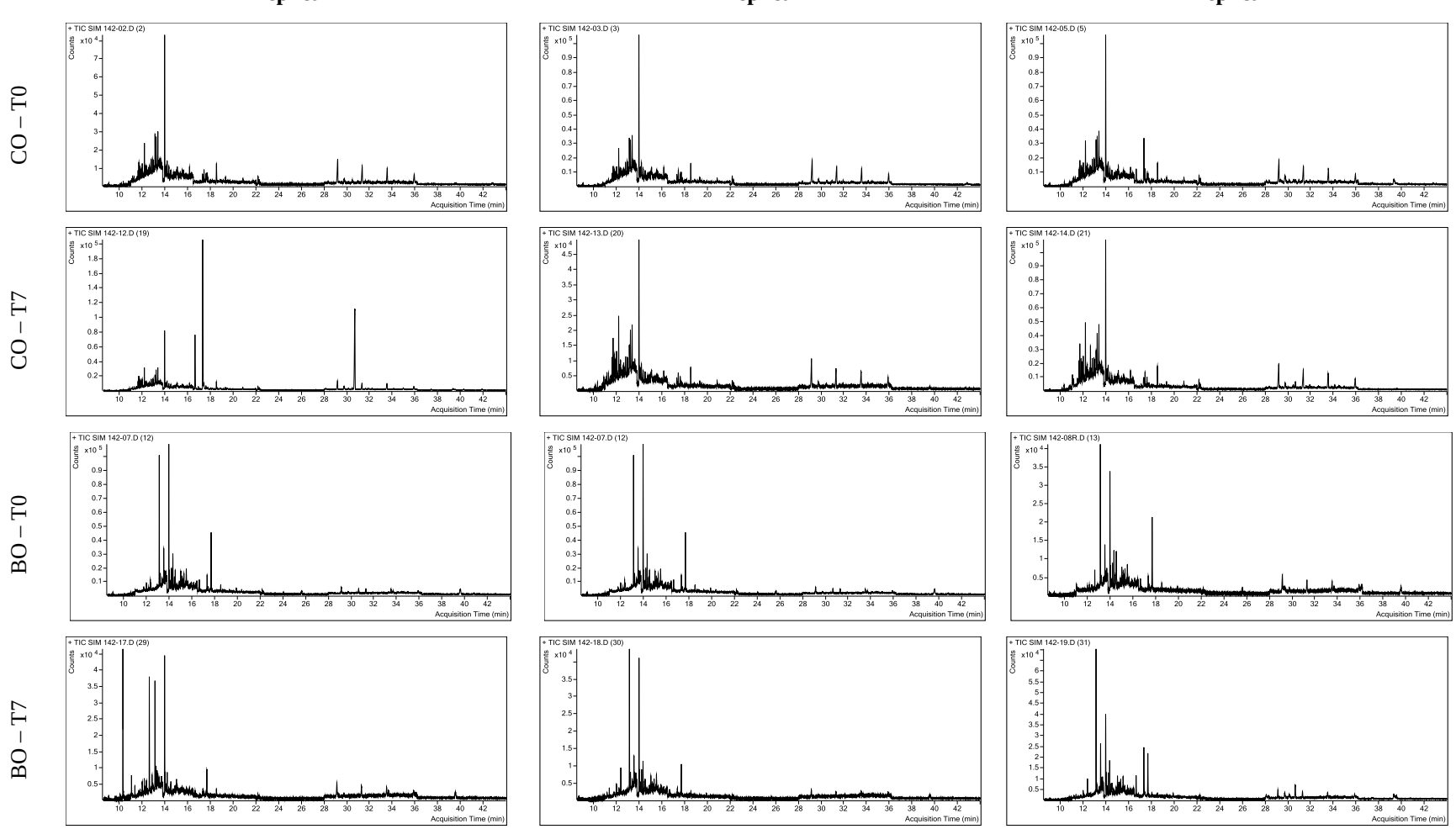
Figura 43 - Perfil cromatográfico dos *n*-alcanos (nC_8 a nC_{40}) do experimento de degradação biótico e abiótico do petróleo nos tempos 0 e 7 dias



Fonte: Autor, 2022.

APÊNDICE 19

Figura 44 - Perfil cromatográfico dos HPAs prioritários do experimento de degradação biótico e abiótico do petróleo nos tempos 0 e 7 dias



Fonte: Autor, 2022.

APÊNDICE 20

Tabela 19 - Médias dos parâmetros geoquímicos monitorados nas unidades de experimentais nos tempos 0 e 7 dias de experimento

AMOSTRA	HTP	UCM	*n*-alcanos	HTP/ UCM	PR	FIT	PR/FIT	*n*-C_{17}	*n*-C_{18}	PR/*n*-C_{17}	FIT/*n*-C_{18}	LEVE	PESADO
O0	4299,76	1535,85	2763,91	2,80	43,47	34,61	1,26	130,73	135,41	0,33	0,26	233,15	1835,73
G0	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
BO0	547,61	267,18	280,42	7,97	0,77	2,13	0,00	2,36	6,73	0,11	0,12	0,72	160,33
BG0	1088,94	249,25	839,70	4,37	1,73	3,24	0,53	1,98	3,19	0,87	1,02	1,33	602,65
BR0	3114,29	310,95	2803,35	9,18	1,58	2,87	0,00	1,08	4,57	-------	0,53	1,38	476,12
O7	2113,44	583,66	1529,78	3,96	22,86	16,78	1,37	64,95	67,12	0,35	0,25	115,17	926,71
G7	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
BO7	1697,35	738,35	959,00	2,30	4,50	8,57	0,50	14,06	22,19	0,38	0,39	10,66	553,83
BG7	363,71	120,44	243,27	3,02	0,00	0,00	0,00	0,00	0,00	-------	-------	1,75	69,06
BR7	256,56	78,22	178,34	4,06	0,00	1,38	0,00	2,87	2,07	-------	-------	2,40	81,23

HTP: Hidrocarbonetos Totais do Petróleo; UCM: Unresolved Complex Misture, Misturas Complexas não Resolvidas; PR: Pristano; FIT: Fitano
Fonte: Autor, 2022.

Tabela 20 - Médias dos parâmetros microbiológicos monitorados nas unidades de experimentais nos tempos 0 e 7 dias de experimento

AMOSTRA	BAC	FUNG	MICRO	PT	LAC	LIP	MNG	C2-3O	AH	DCPIP 0h	DCPIP 24h	DCPIP 48h
O0	9,14	0,00	9,14	247,67	0,16	0,10	-0,95	0,02	-2,07	16,88	23,24	26,60
G0	69,94	0,00	69,94	327,26	0,32	1,05	2,54	0,54	-7,36	11,10	35,93	46,86
BO0	0,04	0,00	0,04	38,19	0,22	-0,14	-3,19	0,06	-8,61	10,31	15,08	14,77
BG0	0,00	0,00	0,00	30,02	0,49	-0,10	-0,82	0,17	-7,43	12,55	15,18	20,55
BR0	0,00	0,00	0,00	33,65	-0,07	2,54	-1,95	0,14	-11,31	12,60	15,48	14,93
O7	5,37	0,00	5,37	245,10	0,35	1,19	-0,69	-0,38	-0,21	17,34	18,34	23,62
G7	5,82	0,14	5,96	524,03	-0,56	13,45	0,07	0,04	5,39	8,19	5,61	30,14
BO7	0,01	0,00	0,01	9,68	-0,04	0,00	0,16	0,01	0,11	12,38	11,01	13,06
BG7	0,00	0,00	0,00	9,29	0,01	0,45	0,11	0,02	0,04	10,31	10,66	24,17
BR7	0,00	0,00	0,00	12,37	0,28	-0,07	0,05	0,02	0,05	10,96	10,69	11,00

PT: Proteínas totais; DCPIP: Dichlorophenolindophenol
Fonte: Autor, 2022.

APÊNDICE 21

Tabela 21 - Perfil de expressão proteica do consórcio microbiano exposto a 8000 mg.L^{-1} de petróleo da bacia do Recôncavo Baiano em 0 dia

Identificação	Comprimento	Peptídeos únicos	Peso molecular (Da)*	N° sequências	N° espectros	Score	Nome da proteína	Gênero
A0A127HR70	98	0	10250,5	1	2	3,2817	10 kDa chaperonin	*Pseudomonas*
A0A3N1KFE1	95	1	9969,3	1	3	7,9826	10 kDa chaperonin	*Stenotrophomonas*
Q818Y9	275	1	31656,2	1	2	2,9039	2,5-diketo-D-gluconic acid reductase	*Bacillus*
ID16	103	0	11727,4	1	4	7,8127	30S ribosomal protein S10	N.E
E2RXV3	101	0	11409,2	1	2	4,366	30S ribosomal protein S14	*Pseudomonas*
A0A127HSG0	156	1	17618,5	2	3	5,0839	30S ribosomal protein S7	*Pseudomonas*
A0A2S9D3G4	231	0	24058,9	1	2	3,3187	50S ribosomal protein L1	*Pseudomonas*
ID130	166	0	17576,4	2	2	3,1594	50S ribosomal protein L10	N.E
ID4506	134	0	14920,8	1	4	8,264	50S ribosomal protein L21	N.E
E2RXU8	110	0	11885,5	1	2	5,1542	50S ribosomal protein L22	*Pseudomonas*
A0A1V2K4B9	121	0	12439,6	1	3	5,1034	50S ribosomal protein L7/L12	*Pseudomonas*
A0A3N1KIZ4	122	1	12517,6	1	7	16,9748	50S ribosomal protein L7/L12	*Stenotrophomonas*
A0A1U9Q3S0	548	1	56952,7	4	9	21,3526	60 kDa chaperonin	*Pseudomonas*
A0A2S9D584	548	1	56867,7	5	13	25,4849	60 kDa chaperonin	*Pseudomonas*
A0A3N1KEA9	549	2	57394,9	3	8	15,5355	60 kDa chaperonin	*Stenotrophomonas*
A0A1U9PTY3	259	0	27435,1	7	23	45,8924	ABC transporter substrate-binding protein	*Pseudomonas*
A0A1U9Q336	261	1	28310,8	4	9	13,7823	ABC transporter substrate-binding protein	*Pseudomonas*
A0A2S9D712	358	0	38594,7	1	2	6,1667	ABC transporter substrate-binding protein	*Pseudomonas*
A0A1V2KIE1	261	0	28288,7	5	10	16,0075	ABC transporter substrate-binding protein	*Pseudomonas*
A0A1U9PSG2	552	0	56325,1	2	6	11,0348	Acetyltransferase component of pyruvate dehydrogenase	*Pseudomonas*
A0A498CH69	467	1	48370,7	1	6	15,1367	Acetyltransferase component of pyruvate dehydrogenase	*Stenotrophomonas*
ID4804	913	0	99208,7	1	2	3,8307	Aconitate hydratase AcnA	N.E

Tabela 21 - Perfil de expressão proteica do consórcio microbiano exposto a 8000 mg.L^{-1} de petróleo da bacia do Recôncavo Baiano em 0 dia (continuação)

Identificação	Comprimento	Peptídeos únicos	Peso molecular (Da)*	N° sequências	N° espectros	Score	Nome da proteína	Gênero
A0A023Y6T6	79	1	8719,3	1	2	2,8569	Acyl carrier protein	*Stenotrophomonas*
A0A127I331	187	0	20420,4	4	9	19,8624	Alkyl hydroperoxide reductase C	*Pseudomonas*
A0A1V2KCV3	200	0	21892	3	8	15,7339	Alkyl hydroperoxide reductase C	*Pseudomonas*
A0A2S9DEL9	250	1	27719,1	2	3	5,5543	Amino acid ABC transporter	*Pseudomonas*
A0A1V2K2C7	300	1	32932,9	2	3	3,0453	Amino acid ABC transporter substrate-binding protein	N.E
ID290	343	0	36592,9	2	3	4,7743	amino acid ABC transporter substrate-binding protein	N.E
ID4467	362	0	38687,3	1	2	4,3006	Asparaginase	N.E
A0A1V2K871	514	0	55240,6	1	4	8,4553	ATP synthase subunit alpha	*Pseudomonas*
A0A2S9DXH9	156	0	16828	2	6	13,4993	ATP synthase subunit beta	*Pseudomonas*
A0A2S9DXM1	458	0	49368,4	3	8	18,0088	ATP synthase subunit beta	*Pseudomonas*
A0A1V2KCA5	156	0	17888,1	1	5	10,001	Bacterioferritin	*Pseudomonas*
A0A498CTL2	194	1	21693,8	1	6	12,4884	Bacterioferritin	*Stenotrophomonas*
ID5011	176	0	19837,9	1	2	4,8067	Bacterioferritin	N.E
A0A2S9DLR2	154	1	16297,5	3	5	9,2803	Biotin carboxyl carrier protein of acetyl-CoA carboxylase	*Pseudomonas*
A0A2S9DFX0	206	0	20902,2	3	9	22,2512	BON domain-containing protein	*Pseudomonas*
A0A3N1K6U0	124	1	12964,8	1	2	3,2683	BON domain-containing protein	*Stenotrophomonas*
A0A1U9PSP9	378	0	39984,4	7	25	60,1227	Branched chain amino acid ABC transporter substrate-binding protein	*Pseudomonas*
A0A1U9PU19	375	0	39607,4	4	9	17,4729	Branched chain amino acid ABC transporter substrate-binding protein	*Pseudomonas*
A0A2S9E5H8	378	1	40003,4	6	21	53,2295	Branched chain amino acid ABC transporter substrate-binding protein	*Pseudomonas*

Tabela 21 - Perfil de expressão proteica do consórcio microbiano exposto a 8000 mg.L^{-1} de petróleo da bacia do Recôncavo Baiano em 0 dia (continuação)

Identificação	Comprimento	Peptídeos únicos	Peso molecular (Da)*	N° sequências	N° espectros	Score	Nome da proteína	Gênero
A0A2S9DJM7	375	1	39681,4	4	8	15,7837	Branched-chain amino acid ABC transporter substrate-binding protein	*Pseudomonas*
A0A2S9DC43	451	1	50194,3	1	3	6,4957	Channel protein TolC	*Pseudomonas*
A0A2S9DFZ4	638	1	68348,3	2	5	9,139	Chaperone protein DnaK	*Pseudomonas*
A0A1V2KIJ5	634	0	71421,6	1	4	9,7847	Chaperone protein HtpG	*Pseudomonas*
A0A1U9PV50	429	0	47709,1	1	2	4,0891	Citrate synthase	*Pseudomonas*
A0A2S9DWL3	70	0	7668,8	1	2	3,3002	Cold-shock protein	*Pseudomonas*
A0A023Y1S8	67	2	7412,8	3	16	32,7628	Cold-shock protein	*Stenotrophomonas*
A0A023Y0S6	68	1	7226,5	1	1	2,4266	Cold-shock protein	*Stenotrophomonas*
ID111	70	0	7633,8	2	6	9,8477	Cold-shock protein	N.E
A0A3N1K5N7	73	1	8504,1	1	3	6,4212	CsbD family protein	*Stenotrophomonas*
A0A2S9DH27	264	1	28621,4	2	5	7,237	Cystine ABC transporter substrate-binding protein	*Pseudomonas*
A0A1V2KC05	93	0	9662,4	3	7	14,008	Dipicolinate synthase	*Pseudomonas*
ID95	124	0	13981,5	2	3	3,5228	DNA binding protein	N.E
ID4011	157	0	17750,1	2	5	10,3049	DNA starvation/stationary phase protection protein	*Pseudomonas*
A0A127HSG9	333	0	36605,3	1	2	3,1893	DNA-directed RNA polymerase subunit alpha	*Pseudomonas*
A0A1U9Q246	309	0	31291,4	1	4	11,7733	Electron transfer flavoprotein subunit alpha	*Pseudomonas*
A0A2S9D8Z0	190	0	21274,7	1	3	4,3056	Elongation factor P	*Pseudomonas*
A0A2S9DBZ4	287	0	30538,3	3	6	10,6196	Elongation factor Ts	*Pseudomonas*
A0A498CCL3	292	1	30742,2	1	3	4,5769	Elongation factor Ts	*Stenotrophomonas*
ID129	397	0	43518,3	2	4	6,528	Elongation factor Tu	N.E
A0A3N1KHA4	115	1	12656,4	1	2	2,7101	Enamine deaminase RidA (YjgF family)	*Stenotrophomonas*

Tabela 21 - Perfil de expressão proteica do consórcio microbiano exposto a 8000 mg.L^{-1} de petróleo da bacia do Recôncavo Baiano em 0 dia (continuação)

Identificação	Comprimento	Peptídeos únicos	Peso molecular (Da)*	N° sequências	N° espectros	Score	Nome da proteína	Gênero
A0A3N1KCE8	130	1	13675	1	2	4,3019	Endoribonuclease L-PSP	*Stenotrophomonas*
ID212	178	0	19242,2	1	2	3,67	F0F1 ATP synthase subunit delta	N.E
ID3352	308	0	33381,3	3	6	9,274	Glutamate/aspartate ABC transporter substrate-binding protein	N.E
A0A2S9DEQ0	468	1	51695,5	1	2	3,3294	Glutamine synthetase	*Pseudomonas*
A0A498C8D5	274	2	29666,7	2	2	4,2299	Heat shock protein HslJ	*Stenotrophomonas*
ID45	90	0	9038,9	2	6	14,5315	HU family DNA-binding protein|HU family DNA-binding protein	*Pseudomonas*
ID228	338	0	36184,3	3	6	9,6433	Ketol-acid reductoisomerase	
ID360	422	0	45001,3	2	6	9,0841	Malate dehydrogenase	N.E
A0A1U9PQP7	260	1	28195,3	2	6	13,9582	Methionine ABC transporter substrate-binding protein	*Pseudomonas*
ID29503	368	1	39035,5	1	3	4,5307	NAD(P)-dependent alcohol dehydrogenase| Zinc-type alcohol dehydrogenase-like protein	*Penicillium*
ID2947	2593	0	288850,7	1	2	2,3493	Non-ribosomal peptide synthetase	N.E
A0A3N1KM80	90	1	9265	1	3	6,2137	Nucleoid protein Hbs	*Stenotrophomonas*
A0A127HRC1	141	0	14909,7	1	2	5,5319	Nucleoside diphosphate kinase	*Pseudomonas*
A0A498CWB4	148	2	15946,3	2	5	10,4843	Nucleotide-binding universal stress UspA family protein	*Stenotrophomonas*
A0A498C307	144	1	15047,6	1	3	6,5765	Osmotically inducible protein OsmC	*Stenotrophomonas*
ID20	83	0	8803,5	1	3	4,407	Outer membrane lipoprotei OprI	N.E
A0A2S9DEK7	438	0	47427,6	2	6	12,4645	Outer membrane porin, OprD family	*Pseudomonas*
A0A2S9DMF5	232	1	24752,6	3	3	4,086	Outer membrane protein OmpW	*Pseudomonas*
A0A3N1KK76	233	1	24968,1	1	2	3,5471	Peptidyl-prolyl cis-trans isomerase	*Stenotrophomonas*

Tabela 21 - Perfil de expressão proteica do consórcio microbiano exposto a 8000 mg.L^{-1} de petróleo da bacia do Recôncavo Baiano em 0 dia (continuação)

Identificação	Comprimento	Peptídeos únicos	Peso molecular (Da)*	N° sequências	N° espectros	Score	Nome da proteína	Gênero
ID777	167	0	18234,2	1	2	4,4694	Peptidyl-prolyl cis-trans isomerase	N.E
ID3452	161	0	16931,3	1	3	8,3845	Peptidylprolyl isomerase	N.E
A0A498BYJ6	220	2	23919,5	2	2	3,2951	Phospholipid transport system substrate-binding protein	*Stenotrophomonas*
ID5584	429	0	45544,3	1	4	6,8085	Phosphopyruvate hydratase	N.E
A0A127HU11	112	0	12304,7	3	7	14,5937	P-II family nitrogen regulator	*Pseudomonas*
A0A498CSD1	283	1	31008,5	1	3	4,4641	Pirin family protein	*Stenotrophomonas*
A0A1U9PS96	332	0	32652,4	1	2	2,158	Poly(3-hydroxyalkanoate) granule-associated protein PhaF	*Pseudomonas*
A0A1U9Q5B2	364	0	40107,6	1	2	3,4219	Polyamine ABC transporter substrate-binding protein	*Pseudomonas*
ID3530	369	0	39925,5	4	13	27,4045	Polyamine ABC transporter substrate-binding protein|putrescine transport system substrate-binding protein	*Pseudomonas*
A0A498C516	359	1	39305,3	1	5	7,0257	Polyketide cyclase/dehydrase/lipid transport protein	*Stenotrophomonas*
A0A1U9Q3L5	326	0	34570,3	2	5	8,6145	Porin	*Pseudomonas*
A0A1U9Q2V0	432	0	46884,2	2	6	14,1734	Porin	*Pseudomonas*
A0A2S9DFW8	186	1	20729,4	2	6	13,6313	Protein GrpE	*Pseudomonas*
A0A2S9DEQ5	159	0	17606,8	2	2	2,7237	Protein-export protein SecB	*Pseudomonas*
ID5060	364	0	39087,3	1	2	2,2311	Pyridoxal phosphate-dependent aminotransferase	N.E

Tabela 21 - Perfil de expressão proteica do consórcio microbiano exposto a 8000 mg.L^{-1} de petróleo da bacia do Recôncavo Baiano em 0 dia (conclusão)

Identificação	Comprimento	Peptídeos únicos	Peso molecular (Da)*	N° sequências	N° espectros	Score	Nome da proteína	Gênero
A0A1V2KCH9	383	0	42129,2	1	2	5,1046	Spermidine/putrescine ABC transporter substrate-binding protein	*Pseudomonas*
ID4726	335	0	35774,4	3	9	15,6621	Substrate-binding domain-containing protein|substrate-binding	*Pseudomonas*
A0A1V2KJ64	590	0	63533,9	1	2	2,7014	Succinate dehydrogenase flavoprotein subunit	*Pseudomonas*
A0A2S9DPP5	388	1	41237,6	2	3	3,7557	Succinate--CoA ligase [ADP-forming] subunit beta	*Pseudomonas*
A0A498BWF6	211	1	21170,3	1	2	4,1335	Superoxide dismutase [Cu-Zn]	*Stenotrophomonas*
ID91	198	0	21945,8	1	2	2,6267	Superoxide dismutase	N.E
ID223	109	0	11847,1	1	2	2,5861	Thioredoxin TrxA	N.E
A0A1V2J6W1	493	0	54377,1	2	5	7,7173	Transcription termination/antitermination protein NusA	*Pseudomonas*
ID2744	266	0	29723,3	1	2	4,2853	Transporter substrate-binding domain-containing protein	N.E
A0A1U9Q1I2	436	0	48264,9	5	8	15,1626	Trigger factor	*Pseudomonas*
A0A498C845	398	4	43521,2	4	15	34,5152	Uncharacterized protein HemY	*Stenotrophomonas*
A0A3N1KEY1	113	1	10404	1	1	4,0731	Uncharacterized protein	*Stenotrophomonas*
A0A498CHW3	167	1	16862,6	1	2	2,771	Uncharacterized protein	*Stenotrophomonas*
A0A498CUX9	129	2	13452,3	2	7	12,4114	Uncharacterized protein	*Stenotrophomonas*
A0A1U9PRZ2	421	1	46075,4	2	4	5,9448	Urea ABC transporter substrate-binding protein	*Pseudomonas*
A0A1U9PWB3	402	1	43670,8	1	2	2,7051	Urea ABC transporter substrate-binding protein	*Pseudomonas*
ID2720	421	0	46105,5	2	4	6,3987	Urea ABC transporter substrate-binding protein	N.E

*O peso molecular previsto calculado a partir da sequência da proteína. Nível de proteína FDR≤0,01. N.E: não encontrado.
Fonte: Autor, 2022

APÊNDICE 22

Tabela 22 - Perfil de expressão proteica do consórcio microbiano exposto a 8000 mg.L^{-1} de petróleo da bacia do Recôncavo Baiano durante 7 dias

Identificação	Comprimento	Peptídeos únicos	Peso molecular (Da)*	N° sequências	N° espectros	Score	Nome da proteína	Gênero
ID54	140	0	16257,8	1	3	5,6851	0S ribosomal protein S6	*Pseudomonas*
A0A3N1KFE1	95	1	9969,3	1	3	5,7019	10 kDa chaperonin	*Stenotrophomonas*
A0A1V2K7K7	407	0	44588,2	1	2	3,1726	2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinol hydroxylase	*Pseudomonas*
E2RXV3	101	0	11409,2	1	5	11,1227	30S ribosomal protein S14	*Pseudomonas*
A0A1V2K4B4	156	0	17618,5	1	4	7,0496	30S ribosomal protein S7	*Pseudomonas*
ID4662	425	0	44780,2	1	2	4,0555	4-aminobutyrate--2-oxoglutarate transaminase	*Pseudomonas*
A0A127HSV4	137	1	15403,5	1	2	3,2669	50S ribosomal protein L16	*Pseudomonas*
A0A1V2K4E4	137	0	15401,6	1	3	5,5288	50S ribosomal protein L16	*Pseudomonas*
A0A2S9D3T1	110	0	11885,5	1	2	4,0252	50S ribosomal protein L22	*Pseudomonas*
A0A3N1KJ16	180	1	20186,6	1	2	3,1904	50S ribosomal protein L5	*Stenotrophomonas*
A0A2S9D3R1	177	0	19109,3	1	1	2,6525	50S ribosomal protein L6	*Pseudomonas*
A0A1U9Q3S0	548	1	56952,7	3	6	17,1058	60 kDa chaperonin	*Pseudomonas*
A0A3N1KEA9	549	2	57394,9	2	7	14,702	60 kDa chaperonin	*Stenotrophomonas*
A0A1U9PTY3	259	0	27435,1	1	3	5,0032	ABC transporter substrate-binding protein	*Pseudomonas*
A0A2S9D9C3	261	3	28365,8	4	7	12,5853	ABC transporter substrate-binding protein	*Pseudomonas*
A0A1V2KIE1	261	0	28288,7	2	5	7,9429	ABC transporter substrate-binding protein	*Pseudomonas*
A0A1U9PSG2	552	0	56325,1	2	4	7,8287	Acetyltransferase component of pyruvate dehydrogenase complex	*Pseudomonas*
A0A2S9DPD6	913	0	99133,7	1	2	3,4081	Aconitate hydratase	*Pseudomonas*
A0A127HXP1	200	0	21901	3	12	25,0021	Alkyl hydroperoxide reductase C	*Pseudomonas*
A0A127I331	187	1	20420,4	5	16	34,2818	Alkyl hydroperoxide reductase C	*Pseudomonas*
A0A2S9DEL9	250	0	27719,1	3	7	12,203	Amino acid ABC transporter	*Pseudomonas*
A0A1V2K404	334	0	36238,9	1	2	2,8765	Aspartate carbamoyltransferase	*Pseudomonas*

Tabela 22 - Perfil de expressão proteica do consórcio microbiano exposto a 8000 mg.L^{-1} de petróleo da bacia do Recôncavo Baiano durante 7 dias (continuação)

Identificação	Comprimento	Peptídeos únicos	Peso molecular (Da)*	N° sequências	N° espectros	Score	Nome da proteína	Gênero
A0A1V2K871	514	0	55240,6	2	5	10,8101	ATP synthase subunit alpha	*Pseudomonas*
A0A1V2JJG8	458	1	49441,4	4	6	12,6442	ATP synthase subunit beta	*Pseudomonas*
ID2862	156	0	17888,1	1	2	3,948	Bacterioferritin	*Pseudomonas*
A0A498CTL2	194	1	21693,8	1	3	5,3305	Bacterioferritin	*Stenotrophomonas*
A0A1V2KIG9	406	0	42838,3	1	1	2,4527	Beta-ketoacyl-[acyl-carrier-protein] synthase I	*Pseudomonas*
A0A1U9PSP9	378	0	39984,4	3	13	29,0013	Branched chain amino acid ABC transporter substrate-binding protein	*Pseudomonas*
A0A2S9DC43	451	1	50194,3	1	4	8,9963	Channel protein TolC	*Pseudomonas*
A0A2S9DFZ4	638	1	68348,3	3	5	8,5831	Chaperone protein DnaK	*Pseudomonas*
A0A2S9DPT3	429	0	47680,1	1	2	3,5428	Citrate synthase	*Pseudomonas*
A0A023Y1S8	67	1	7412,8	1	2	4,9225	Cold-shock protein	*Stenotrophomonas*
A0A3N1K5N7	73	1	8504,1	1	2	4,3604	CsbD family protein	*Stenotrophomonas*
A0A1U9PRV6	264	0	28357,4	1	2	4,3697	Cystine ABC transporter substrate-binding protein	*Pseudomonas*
A0A1V2KI48	157	0	17750,1	2	5	10,5898	DNA starvation/stationary phase protection protein	*Pseudomonas*
A0A127HSG9	333	0	36605,3	3	5	10,7364	DNA-directed RNA polymerase subunit alpha	*Pseudomonas*
A0A2S9DBZ4	287	0	30538,3	1	1	2,7061	Elongation factor Ts	*Pseudomonas*
A0A1U9Q4N7	397	1	43535,2	2	7	15,5327	Elongation factor Tu	*Pseudomonas*
A0A3N1KCE8	130	1	13675	1	2	4,2091	Endoribonuclease L-PSP	*Stenotrophomonas*
A0A127HV08	468	1	51623,5	5	11	18,0007	Glutamine synthetase	*Pseudomonas*
A0A2S9DEQ0	468	1	51695,5	4	8	16,9787	Glutamine synthetase	*Pseudomonas*
A0A498CI70	131	1	13999,9	1	1	2,8931	Glycine cleavage system H protein	*Stenotrophomonas*
ID26389	933	0	103377,6	1	2	3,5382	Heat shock protein	*Penicillium*
A0A498C8D5	274	2	29666,7	2	5	9,6194	Heat shock protein HslJ	*Stenotrophomonas*
A0A127HRK5	338	0	36170,2	3	9	19,8638	Ketol-acid reductoisomerase (NADP(+))	*Pseudomonas*
A0A127HVG6	422	0	45039,3	1	4	7,9179	Malate dehydrogenase	*Pseudomonas*

Tabela 22 - Perfil de expressão proteica do consórcio microbiano exposto a 8000 mg.L^{-1} de petróleo da bacia do Recôncavo Baiano durante 7 dias (conclusão)

Identificação	Comprimento	Peptídeos únicos	Peso molecular (Da)*	N° sequências	N° espectros	Score	Nome da proteína	Microrganismo
ID5594	634	0	71421,6	1	2	6,0163	Molecular chaperone HtpG	*Pseudomonas*
A0A3N1KM80	90	1	9265	1	2	4,8271	Nucleoid protein Hbs	*Stenotrophomonas*
ID1	112	0	12304,7	2	8	14,1907	Peroxiredoxin	*Pseudomonas*
A0A498C516	359	1	39305,3	1	4	5,6861	Polyketide cyclase/dehydrase/lipid transport protein	*Stenotrophomonas*
A0A1U9Q2V0	432	0	46884,2	2	7	17,3401	Porin	*Pseudomonas*
A0A2S9DI29	326	0	34537,3	2	5	8,6876	Porin	*Pseudomonas*
A0A2S9DFW8	186	1	20729,4	1	2	3,8712	Protein GrpE	*Pseudomonas*
ID3530	369	0	39925,5	2	6	14,5877	Putrescine transport system substrate-binding protein	*Pseudomonas*
A0A2S9DZM8	396	0	42523,4	1	3	4,2795	S-adenosylmethionine synthase	*Pseudomonas*
A0A1U9Q3G8	198	0	21961,8	2	4	6,8224	Superoxide dismutase	*Pseudomonas*
A0A2S9DFU9	493	0	54433,2	1	2	3,6197	Transcription termination/antitermination protein NusA	*Pseudomonas*
ID2342	436	0	48416	1	2	3,867	Trigger factor	*Pseudomonas*
A0A2S9D908	83	0	8803,5	1	2	3,3094	Uncharacterized protein	*Pseudomonas*
A0A3N1KEY1	113	1	10404	1	1	3,1143	Uncharacterized protein	*Stenotrophomonas*
A0A498CTS0	272	2	30064,9	2	4	8,7484	Uncharacterized protein	*Stenotrophomonas*
A0A2S9DZR8	199	0	21236,8	1	4	9,2668	YceI domain-containing protein	*Pseudomonas*

*O peso molecular previsto calculado a partir da sequência da proteína. Nível de proteína FDR≤0,01

Fonte: Autor, 2022

APÊNDICE 23

Tabela 23 - Perfil de expressão proteica do consórcio microbiano cultivado em 10 mL.L^{-1} de glicerol no tempo 7 dias

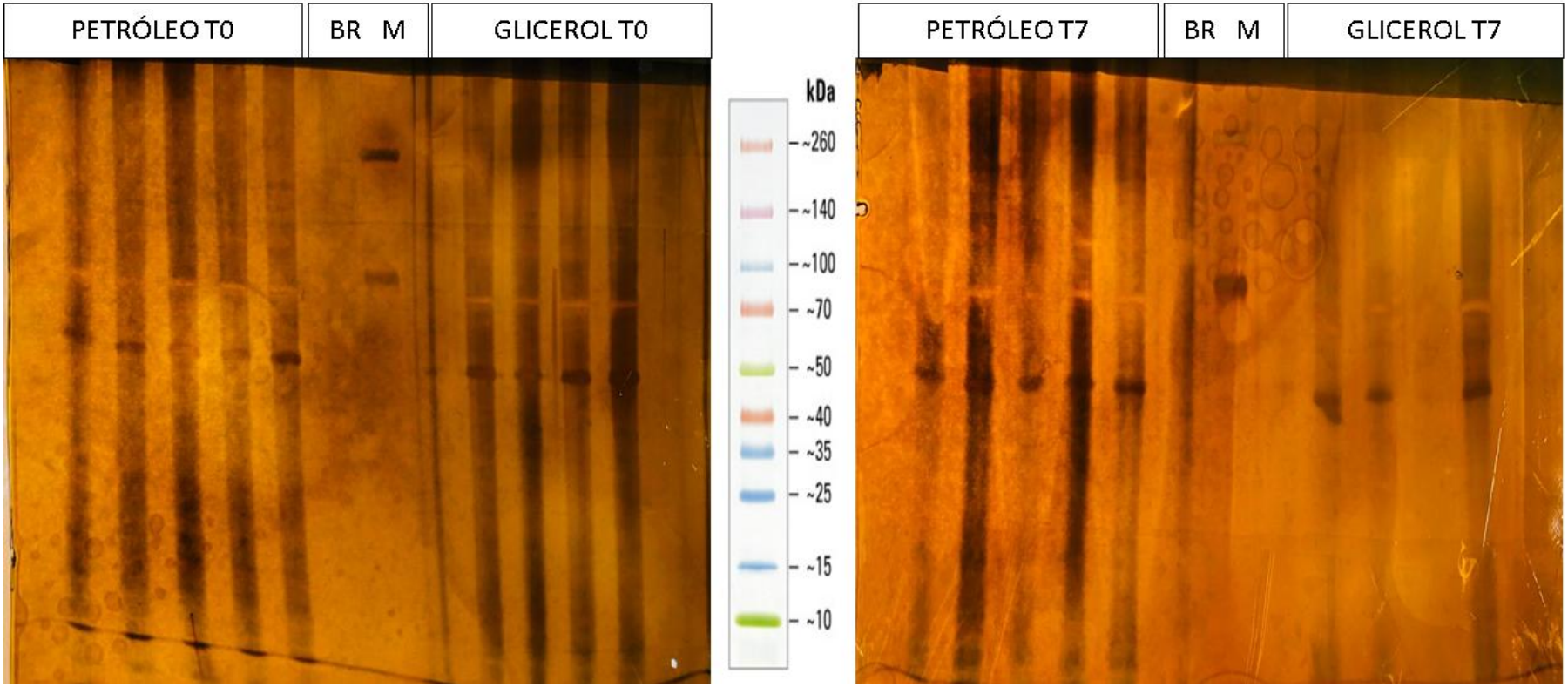
Identificação	Comprimento	Peptídeos únicos	Peso molecular (Da)*	N° sequências	N° espectros	Score	Nome da proteína	Microrganismo
A0A2S9DUE4	187	0	20391,4	1	2	3,3019	Alkyl hydroperoxide reductase C	*Pseudomonas*
A0A498CQM4	762	1	84631,8	1	3	5,6303	Arginine decarboxylase	*Stenotrophomonas*
Q814W0	505	1	54987,8	1	3	6,075	ATP synthase subunit alpha	*Bacillus*
A0A498CH71	515	0	55410,9	1	3	6,6764	ATP synthase subunit alpha	*Stenotrophomonas*
Q818A3	377	1	41232,3	1	5	9,8961	Cystathionine beta-lyase	*Bacillus*
Q814C4	395	2	42893,9	2	4	6,3612	Elongation factor Tu	*Bacillus*
Q815K8	431	2	46325,3	2	6	11,5015	Enolase	*Bacillu*
A0A1V2KBX4	468	0	51623,5	1	3	6,6106	Glutamine synthetase	*Pseudomonas*
ID24455	288	0	32599,6	1	8	16,1922	Inorganic pyrophosphatase	*Penicillium*
Q813P2	147	1	16651,2	1	6	10,6937	Iron-binding ferritin-like antioxidant protein / Ferroxidase	*Bacillus*
A0A1U9PSC6	387	0	43378,2	1	3	5,1788	Ornithine decarboxylase	*Pseudomonas*
A0A2S9DI29	326	1	34537,3	3	9	27,9271	Porin	*Pseudomonas*
Q81HW6	215	1	23094,2	1	2	3,489	Probable transaldolase 1	*Bacillus*
Q812J2	180	1	21047,1	1	5	9,8441	Ribosome hibernation promoting factor	*Bacillus*
Q81A24	180	1	20311,8	1	4	7,7847	Spore coat protein E	*Bacillus*
Q81IE4	192	1	20771,3	1	2	3,3691	Tellurium resistance protein terD	*Bacillus*
Q817L8	104	1	11452,9	1	2	2,7242	Thioredoxin	*Bacillus*
ID36318	448	1	50035,5	1	4	7,2879	Tubulin alpha-2 chain	*Penicillium*
ID25566	438	0	47228,5	1	3	5,8741	Unnamed protein product	*Penicillium*

*O peso molecular previsto calculado a partir da sequência da proteína. Nível de proteína FDR≤0,01

Fonte: Autor, 2022.

APÊNDICE 24

Figura 45 – Fotodocumentação de géis de proteínas pelo método SDS-PAGE, corados com nitrato de prata. A esquerda visualização de perfis de bandas de proteínas a 60 µg em zero dia de incubação. A direita visualização de perfis de bandas de proteínas a 60 µg em sente dias de incubação



BR= Branco do experimento; M= Marcador de proteína; T0=Tempo 0 dia; T7=Tempo 7 dias

Fonte: Autor, 2022

APÊNDICE 25

Figura 46 - Foto dos mesocosmos após 7 dias de incubação

Glicerol e consórcio | Petróleo e consórcio | Petróleo sem consórcio

Fonte: Autor, 2022

APÊNDICE 26

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CONTATO

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IDIOMA

- ✓ Português -Materno
- ✓ Inglês - Intermediário

HABILIDADES

- ✓ Word - Intermediário
- ✓ PowerPoint - Intermediário
- ✓ Excel - Intermediário
- ✓ Software R - Básico
- ✓ Minitabl - Básico
- ✓ Mega X - Básico

FORMAÇÃO

Doutorado em Geoquímica: Petróleo e Meio Ambiente
Universidade Federal da Bahia | 2017 - 2022

Mestrado em Geoquímica: Petróleo e Meio Ambiente
Universidade Federal da Bahia | 2014 - 2016

Especialização em Ecologia e Intervenções Ambientais
Centro Universitário Jorge Amado | 2013 – 2014

Bacharelado e licenciatura em Ciências Biológicas
Centro Universitário Jorge Amado | 2009 - 2014

PERFIL

Bióloga com histórico em microbiologia e biotecnologia ambiental, ex - representante estudantil no POSPETRO e ex - membro da Comissão Interna de prevenção de acidentes no LEPETRO. Busca por evolução profissional e adora compartilhar experiências.

EXPERIÊNCIA PROFISSIONAL

2017-2021 **Bolsista FAPESB** (Doutorado em Geoquímica: petróleo e meio ambiente)
Universidade Federal da Bahia
Suporte técnico em treinamento de biossegurança, desenvolvimento de protocolos de biologia molecular e clásica, extração de DNA e amplificação em PCR convencional, extração de proteínas, quantificação de DNA e proteínas por eletroforese, espectrofotômetro e fluorímetro e gestão de alunos de iniciação científica.

Out - 2019 **Extensão Universitária** (Projeto CBAB-CABIO)
Universidade Nacional de Tucumán – Argentina
Coleta e produação de fungos em fermentação sólida e líquida. Produção de fungos em escala de biorreator. Teste de antimicrobiano e quantificação de proteínas.

Set - 2015 **Extensão Universitária** (Projeto Newton)
Newcastle University – Inglaterra
Extração de DNA total e amplificação em PCR convencional, quantificação de DNA por eletroforese e fluorímetro, limpeza e preparo de amostra para sequenciamento de nova geração.

2014 - 2016 **Bolsista FAPESB** (Mestrado em Geoquímica: petróleo e meio ambiente)
Universidade Federal da Bahia
Isolamento e preservação de fungos e bactérias, encapsulamento de microrganismos, ensaios antagônicos, construção de protótipo de biorreator, manipulação de microscópio óptico e manipulação de aparelho soxhlet

2012 - 2014 **Estágiaria** (Apoio técnico)
Instituto do Meio Ambiente e Recursos Hídricos
Monitoramento das atividades do projeto de corredores ecológicos, processos administrativos do sistema e gestão de unidade de conservação.

2011 - 2012 **Iniciação Científica** (Voluntária)
Centro Universitário Jorge amado em parceria com a Universidade Livre das Dunas e Restinga de Salvador
Caracterização morfológica e monitoramento de visitantes florais de *Epidendrum cinnabarinum*.

2010 - 2011 **Estágiaria** (Voluntária)
Centro Universitário Jorge Amado
Apoio técnico a aulas práticas em laboratório de bioquímica e apoio técnico a aulas práticas em laboratório de morfologia e anatomia vegetal.