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Review

Petroleum Hydrocarbon Catabolic Pathways as Targets for Metabolic Engineering Strategies for Enhanced Bioremediation of Crude-Oil-Contaminated Environments

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Abstract: Anthropogenic activities and industrial effluents are the major sources of petroleum hydrocarbon contamination in different environments. Microbe-based remediation techniques are known to be effective, inexpensive, and environmentally safe. In this review, the metabolic-target-specific pathway engineering processes used for improving the bioremediation of hydrocarbon-contaminated environments have been described. The microbiomes are characterised using environmental genomics approaches that can provide a means to determine the unique structural, functional, and metabolic pathways used by the microbial community for the degradation of contaminants. The bacterial metabolism of aromatic hydrocarbons has been explained via peripheral pathways by the catabolic actions of enzymes, such as dehydrogenases, hydrolases, oxygenases, and isomerases. We proposed that by using microbiome engineering techniques, specific pathways in an environment can be detected and manipulated as targets. Using the combination of metabolic engineering with synthetic biology, systemic biology, and evolutionary engineering approaches, highly efficient microbial strains may be utilised to facilitate the target-dependent bioprocessing and degradation of petroleum hydrocarbons. Moreover, the use of CRISPR-*cas* and genetic engineering methods for editing metabolic genes and modifying degradation pathways leads to the selection of recombinants that have improved degradation abilities. The idea of growing metabolically engineered microbial communities, which play a crucial role in breaking down a range of pollutants, has also been explained. However, the limitations of the *in-situ* implementation of genetically modified organisms pose a challenge that needs to be addressed in future research.

Keywords: crude oil contamination; bioremediation; synthetic biology; catabolic pathways analysis; metabolic pathway engineering

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1. Introduction

Crude oil contamination is an emerging environmental concern on a global scale due to inadvertent oil leakage [1]. Oil spillage and discharges frequently happen as a consequence of explosion incidents during oilfield drilling; leakage from oil and gas pipelines and reservoirs, fuel tankers, and well waxing; and during overhauls of refineries and petrochemical manufacturing equipment [2,3]. Crude oil is a heterogeneous mixture primarily composed of carbon (83–87%) and hydrogen (10–14%) atoms. However, fractions of nitrogen (0.1 to 2%), sulphur (0.05 to 6.0%), and oxygen (0.05 to 1.5%) compounds (NSO) are also found [4,5]. According to its structural complexity, crude oil is classified into four

major types: paraffins (15–60%), naphthenes (30–60%), aromatics (3–30%), and asphaltenes (2–25%). The simplest form of crude oil with at least one saturated carbon atom ring is naphthenes [6], which include single-ring aromatic compounds, such as benzene, toluene, ethylbenzene, and xylene (BTEX), and multi-ring polycyclic aromatic hydrocarbons (PAHs), with various alkyl groups making up the aromatic fraction [7]. The highly complex carbon structure of resins and asphaltenes, with many additional NSO atom moieties, makes them the most persistent components of crude oil [8].

Crude oil and its products damage water and air quality and also reduce soil fertility [9,10]. Crude oil can harm plants by clogging soil pores, reducing soil aeration and water permeability, which can have both ecological and toxicological effects and disrupt the soil's natural structure [11,12]. Aside from these environmental concerns, one of the most serious consequences of such anthropogenic emissions is current global climate change. Crude oil contamination not only impacts different ecosystems but also influences global socio-economic status [13]. The two most historic marine crude oil spills with devastating environmental consequences were during the Gulf War in 1991 and the Deepwater Horizon disaster in 2010. Cleaning up such spillage may cost from USD 2.4 billion to USD 61 billion, depending on the penalties and effects on natural and economic resources [14]. Furthermore, even at low concentrations, crude oil substances can cause fatal alterations in genetic material due to their persistence and biological toxicity [15]. Hydrocarbon substances may cause damage to the respiratory, circulatory, nervous, sensory, and immune systems of the human body [16]. Extreme weather phenomena, including cold waves, heat waves, cyclones, floods, and earthquakes, also disrupt food and water supplies. To overcome such difficulties, techniques such as phytoremediation and rhizoremediation are employed to degrade hydrocarbon contaminants and could be used as a biomass-generating source [17]. Many plant–microbe-assisted bioremediation studies have been carried out in order to efficiently remediate crude oil and heavy metal contaminants from various polluted sites [18]. In one of the studies, *Melia azedarach* plants growing in soil contaminated with benzo[a]pyrene (BaP) degraded 88% of the substantial amount of BaP within 60 days of treatment under greenhouse conditions, as well as degrading 68.22% in bulk soil. As a result of the plant–microbe interaction, BaP degradation was increased by 15% [18]. A bacterial strain, *Comamonas* sp. KD7, when associated with the plant *Trifolium repens* L, was discovered to improve dioxin-polluted soil clean-up [19]. Grass species, such as *Cymbopogon ambiguus*, *Brachiaria decumbens*, and *Microlaena stipoides*, enhanced the capacity of indigenous microorganisms to eliminate crude oil pollution in soil without applying external supplements [20]. Another study used the ornamental plant *Tagetes erecta* L in conjunction with the non-pathogenic *K. pneumoniae* AWD5 strain to determine the degradation of pyrene-contaminated soil (200 mg/kg). The degradation rate was 68.61% after 60 days of experiments [21].

Raymond et al. [22] published their first research on the *in-situ* bioremediation treatment of groundwater contaminated with petroleum hydrocarbons due to pipeline leakage in Ambler, Pennsylvania, in 1972. Bioremediation is a sustainable method facilitated by living organisms to break down and remove environmental pollutants [7]. Microbial candidates are potential contributors in bioremediation procedures due to their short and simple genome size, quick regenerating time, and ability to adapt to harsh conditions [23]. A bacterial consortium of *Pseudomonas pseudoalcaligenes* and two strains of *Erythrobacter citreus* degraded 84.1% of hydrocarbon contaminants [24]. In another study, a bacterial consortium of *P. aeruginosa*, *B. subtilis*, and *A. lwoffi* showed the maximum degradation of 88.5% of crude oil in 28 days [25]. Microbial species, such as *Bacillus*, *Mycobacterium*, *Acinetobacter*, and *Micrococcus*, were proven to degrade asphaltenes in different concentrations ranging from 2500 to 5000 mg/L in liquid medium [26,27]. Biological techniques include the microbial (fungal or bacterial) breakdown of contaminants [28]; bioaugmentation and biostimulation to enhance bioremediation [29]; synthesis of value-added biochemical substances; use of plants, particularly those that are fast-growing with large biomass production; and the combined action of plants and microbes to remediate crude oil contamination [30].

Catabolic enzymes are primarily responsible for the microbial degradation of petroleum hydrocarbons [31]. Both aerobic and anaerobic degradation processes involve several enzymatic reactions of contaminants, such as oxidation, reduction, hydrogenation, and dehydrogenation [32]. Systemic biology encompassed all approaches to metabolic pathway engineering, multi-omics techniques (genomic, proteomic, and transcriptomic), and computational applications to investigate all critical processes, and optimise, forecast, and assess the metabolic activities of microorganisms in the relevant environment [33]. Recent advancements in genome editing tools (TALEN, CRISPR-*cas*, and ZFN) [34] and recombinant DNA technology have facilitated the modification of microbial strains for their metabolic pathways to neutralise single or numerous contaminants at the same time [35,36]. Synthetic biology utilises systems biology tools to reconstruct synthetic metabolic routes for the enhancement of the bioremediation process. However, the expense, time constraints, human resources, and goals of the project are needed to be considered when selecting techniques to employ such approaches [37].

Previous reviews have strongly emphasized the use of genetic techniques for microbe-mediated bioremediation. Kotoky et al. [38] reviewed the plant–microbe-assisted rhizoremediation of PAH-contaminated soil. Azad et al. [39] examined the genetically engineered microorganisms involved in the bioremediation of petroleum hydrocarbons and heavy metal contamination. Similarly, in their review, Koshlaf et al. [40] discussed the different bioremediation approaches along with the factors influencing the bioremediation process. These reviews have provided excellent insights into the process of the microbial degradation of hydrocarbons. Here, we have presented an elaborate discussion of the potential of the metabolic engineering of hydrocarbon degradation pathways. Moreover, in contaminated environments, competition from indigenous microorganisms for nutrients, including growth factors, could affect the engineered organisms. There are also some threats to the direct application of genetically modified organisms (GMOs) in the environment due to their highly resistant properties. Furthermore, most of the microorganisms cannot degrade several crude oil components simultaneously [41,42]. As a result, degrading different fractions may necessitate separate machinery or the use of an efficient microbial consortium. Therefore, this review describes aspects of metabolic-pathway-engineering approaches for the significant hydrocarbon remediation of soil based on recent advances in molecular techniques. This review also provides a better understanding of several systems biology approaches integrated with metabolic engineering techniques. Furthermore, this article discusses computational applications for developing sustainable bacterial remediation techniques for future petroleum hydrocarbon contaminations of soil. The study period of this review is mainly from reports after 2000; yet, some pioneering work has been retained for their significant inputs.

This review comprises of four major areas, which are as follows: (1) The microbe-assisted bioremediation of crude-oil-contaminated environments, providing brief details of the microorganisms involved in the bioremediation of crude oil and the factors affecting the bioremediation process. This section also includes information regarding the classical pathways of crude oil degradation. (2) Metabolic pathway engineering for the bioremediation of crude oil contamination, which consists of the different approaches of metabolic engineering via a selection of microorganisms and their transcriptional modifications, and several computational applications to predict toxicity and pathway modification have been discussed. (3) Metabolic engineering studies integrating systems biology, describing the different "omics" approaches. Additionally, computational applications along with the limitations have been provided. Finally, (4) synthetic biology approaches in the field of metabolic engineering, demonstrating the use of genetic engineering tools, the limitations of GMO applications, and risk assessments.

2. Microbe-Assisted Remediation of Crude-Oil-Contaminated Environment

With all the shortcomings of other techniques of remediation, the complete or partially effective removal of crude oil is still a challenge [43–45]. The microbial degradation of

complex and diverse crude oil molecules requires specific enzymes for a specific class of compounds [46,47]. Depending on the bacterial species and community, the resulting catabolites are either utilised by the bacteria themselves or released into the environment for further degradation by the other bacteria in the community [48]. The sole determining factor critical to the degradation is the survival of the bacteria in a medium with high crude oil contamination [49]. Due to their capacity to degrade a variety of crude oil components, bacteria are considered to be the most effective degraders of crude oil [50–52]. According to the research by El-Liethy et al. [53] the rate of crude oil degradation by the bacterial strain *Enterobacter hormaechei* was 0.6% in a minimal medium, but it accelerated to 70.7% when the medium was biostimulated with peptone. A bioremediation experiment carried out with biosurfactant-producing *Pseudomonas aeruginosa* showed a maximum of 68.3% degradation of *n*-hexadecane in 60 days [54]. Alkanes with different molecular chains may be degraded by the *Acinetobacter* strain DSM17874 using degrading genes, such as *alkB* and *AlmA* [55]. *Pseudomonas nitroreducens* efficiently degraded 70% of paraffinic contaminants in a short period of 10 days [56]. However, a consortium can attain better degradation of crude oil by the availability of a broader range of enzymes acting upon crude oil compounds, as well as the co-existence of multiple bacteria that assist in the formation of higher metabolic networks, which can interact with the persistent compounds and carry out the process of biodegradation [52,57]. A microbial consortium consisting of *Actinotalea ferrariae*, *Arthrobacter ginsengisoli*, *Dietzia cinnamea*, *Dietzia papillomatosis*, and *Pseudomonas songnensis* showed a degradation of crude oil ranging from 73.6 to 69.3% in soil with 1–10% crude oil contamination [58]. A study of in situ rhizoremediation revealed that a consortium of *Pseudomonas aeruginosa* and *P. plecoglossicida* degraded 56.14% of petroleum engine oil after 120 days when planted with wheat plants [59].

Bacteria, such as *Acidobacter*, *Alteromonas*, *Arthrobacter*, *Burkholderia*, *Dietzia*, *Enterobacter*, *Kocuria*, *Marinobacter*, *Mycobacterium*, *Pandoraea*, *Pseudomonas*, *Staphylococcus*, *Streptobacillus*, *Streptococcus*, and *Rhodococcus*, play crucial roles in the degradation of crude oil [60,61]. *Acinetobacter*, *Aquabacteriumalong*, *Brevundimonas*, and *Pseudomonas* predominated in crude-oil-contaminated soil by bioremediating 49.3% (1756 mg/kg) of HMW crude oil components with food waste composting [62]. Similarly, *Exiguobacterium aurantiacum* and *Burkholderia cepacian* could degrade 52.93% and 51.37% of crude oil (1%) in 15 days [63], respectively. Similarly, obligate hydrocarbonoclastic bacteria, such as Nesiotobacter, *Nitratireductor*, *Acinetobacter*, *Marinobacter*, *Pseudoalteromonas*, and *Sphingomonas*, have very low abundances in uncontaminated soil [64], otherwise rising with an increase in the contamination [65–67]. *Sphingomonas* strain 4c can break down 87.2% (100 mg/L) of the fluorene in 7 days [68]. *Nesiotobacter exalbescens* COD22 isolated from a marine environment can degrade 92% of hydrocarbon contaminants under high-pressure conditions. However, at a normal pressure rate, the degradation was found to be slowed down by a rate of 87.5% [69]. The symbiotic association of *Talaromyces* species and *Acinetobacter baumannii* can potentially degrade a complete alkane fraction and about 80% of the total petroleum hydrocarbon contamination within 14 days in culture conditions at a pH of 9 [70]. The bacterial functions that allow them to thrive in such extremely polluted conditions are their unique genetic features and catabolic attributes [71]. A consortium consisting of *Aeromonas hydrophila*, *Alcaligenes xylosoxidans*, *Gordonia* sp., *Pseudomonas fluorescens*, *Pseudomonas putida*, *Rhodococcus equi*, *S. maltophilia*, and *Xanthomonas* sp. was able to potentially degrade 89% of diesel oil from heavily contaminated field soil [62].

Crude oil has been an integral part of the marine environment for millions of years, and microbes use it as an energy source. PAHs are ubiquitous in the marine environment and enter through chronic or acute pollution events, such as oil spills. The fate of PAHs after entering the marine environment is determined by several factors, such as sinking, sedimentation, resuspension, volatilisation, photodegradation, and biodegradation [72]. Over 175 prokaryotic genera in 7 phyla of bacteria and archaea and a similar number of fungal genera have been identified that can use hydrocarbons as their sole carbon source [73]. Some commonly reported genera for oil degradation are *Alcanivorax*,

Cycloclasticus [74], *Fundibacter* [75], and *Oleispira* [76]. *Cycloclasticus* can use pyrene, naphthalene, 2-methylnaphthalene, 2,6-dimethylnaphthalene, biphenyl, fluorene, acenaphthalene, dibenzofuran, phenanthrene, or anthracene as a sole carbon source [77]. A recent study in Mediterranean waters revealed that the microbial communities in pristine, unpolluted locations responded very differently than those inhabiting chronically polluted sites. The latter showed a faster degradation response when accidental oil pollution occurred [73]. Mixed bacterial species of *Pseudomonas* sp. and *Bacillus* sp. degrade 80.64% of crude oil and 76.30% of alkanes in oily wastewater. Another study by Chen et al. [78] showed that the bacterial consortium consisting of *Exiguobacterium* sp., *Pseudomonas aeruginosa* sp., *Alcaligenes* sp., *Alcaligenes* sp., and *Bacillus* sp., when isolated from the coastal area, could degrade up to 70% of crude oil in 7 days. A marine bacterial sp. of *Pseudomonas* sp. sp48 can degrade a wide variety of aromatic and aliphatic hydrocarbons by up to 79% in culture conditions [79]. The degradation of different PAHs by bacterial strains has been listed in Table 1, along with other details, such as the specific contaminants they degrade and the environment from where they were isolated.

Table 1. Degradation of PAHs by various microorganisms in soil environment (with their concentrations and degradation rates).

Sl. No.	Microorganism	Specific Polyaromatic Hydrocarbon (PAHs)	Degradation Percentage (%)	Concentration (mg/L)	Environment from Which Bacteria Are Isolated	References
1	*Methylobacterium*, *Burkholderia* and *Stenotrophomonas*.	Phenanthrene	94.5	500	Heavy-metal- and PAH-contaminated sites	[80]
		Pyrene	17.8	10		
2	*Pseudomonas brassicacearum* strain MPDS	Naphthalene		50	PAH-contaminated sites	[81]
		Fluorene	40.3	5		
		Dibenzofuran	65.7	5		
		Dibenzothiophene	32.1	5		
3	*Pseudomonas aeruginosa*	Fluorene	96	20	Hydrocarbon-contaminated sites	[82]
		Phenanthrene	50	20		
		Pyrene	41	20		
4	*Zhihengliuella* sp. ISTPL4	Phenanthrene	87	250	Contaminated frozen sites	[83]
5	*Bacillus pumilus*	Fluoranthene	76.03	500	Oil-spill sites	[84]
		Phenanthrene	87.98	500		
6	*Bacillus simplex*	Fluoranthene	86.89	500	Oil-spill sites	[84]
		Phenanthrene	95.13	500		
7	*Pseudomonas stutzeri*	Fluoranthene	64.97	500	Oil-spill sites	[84]
		Phenanthrene	86.32	500		
8	*Bosea*, *Arthrobacter*, *Paenibacillus*, *Bacillus*, and *Rhodococcus*	Pyrene	100		Farmland	[85]
		Benzo [a]pyrene	26.9–71.5			
9	*Sphingobium* sp. NS7	Pyrene	5.6		Farmland	[85]
		Benzo[a]pyrene	8.6			
10	*Cellulosimicrobium cellulans* CWS2	Benzo[a]pyrene	78.8	10	PAH-contaminated soil	[86]
11	*Pseudomonas* sp.	Naphthalene	95.3	100	Plants from PAH-contaminated site	[87]
		Fluoranthene	87.9	100		
		Phenanthrene	90.4	100		
		Pyrene	6.9	100		

Table 1. *Cont.*

Sl. No.	Microorganism	Specific Polyaromatic Hydrocarbon (PAHs)	Degradation Percentage (%)	Concentration (mg/L)	Environment from Which Bacteria Are Isolated	References
12	*Stenotrophomonas* sp.	Naphthalene	98.0	100	Plants from PAH-contaminated sites	[87]
		Fluoranthene	83.1	100		
		Phenanthrene	87.8	100		
		Pyrene	14.4	100		
		Benzo[a]pyrene	1.6	10		
13	*Micrococcus luteus*	Naphthalene	68.7	1	Petroleum-contaminated soil	[88]
		Fluoranthene	61.4	1		
		Phenanthrene	62.9	1		
		Pyrene	61.3	1		
14	*Kocuria rosea*	Naphthalene	59.8	1	Petroleum-contaminated soil	[88]
		Fluoranthene	53.8	1		
		Phenanthrene	54.6	1		
		Pyrene	53.3	1		
15	*Serratia* sp. PW7	Pyrene	51.2	50	Plant from contaminated sites	[89]
16	*Staphylococcus nepalensis*	Pyrene	93.25	50	Diesel-contaminated soil	[90]
17	*Sphingomonas koreensis* ASU-06	Naphthalene	100	100	Soil from oil refinery	[91]
		Phenanthrene	99	100		
		Pyrene	92.7	100		
		Anthracene	98	100		
18	*Streptomyces* sp.	Fluoranthene	92	100	Bitumen-contaminated soil	[92]
		Phenanthrene	80	100		
		Pyrene	28	100		
		Anthracene	78.2	100		
19	*Ochrobactrum* sp. VA1	Anthracene	88	3	Petroleum- and coal-contaminated sites	[93]
		Phenanthrene	98	3		
		Naphthalene	90	3		
		Fluorene	97	3		
		Pyrene	84	3		
		Benzo[e]pyrene	50	1		
		Benzo[k]fluoranthene	57	1		

2.1. Regulatory Factors Involve in Microbial Degradation of Crude Oil

Microbes are primarily responsible for the biodegradation of crude oil in the contaminated environment; however, several biotic and abiotic factors also influence the efficacy of bioremediation approaches, such as soil oxygen content, soil type, pH, temperature, nutrient availability, water content, and the concentration of crude oil and its bioavailability in the existing environment [94].

The bioavailability of crude oil components in a soil environment is an essential factor in regulating biodegradation rate [95]. Though the increase in the concentration of crude oil negatively affects the degradation rate, the degradation of aromatic compounds and linear hydrocarbons occurs discretely [96]. After the rapid initial rate of crude oil degradation, the residue becomes partially diffused to the surrounding solid surfaces, reducing its bioavailability, phosphorus and nitrogen contents and degradation rate at later stages [97]. The improper disposal of crude oil with high concentrations of volatile hydrocarbons and sludge (15−20%) can harm the microbial population and hinder the biodegradation

of the oil [98,99]. Temperature has multiple effects in the bioremediation process, as it influences the rate at which the crude oil is degraded by the microorganisms, affecting the composition of the whole soil microbiome [100]. The chemical and physical properties of crude oil are also affected by temperature variations. The soil–water partition coefficient decreases with increasing temperature in soil with high moisture content, and contaminant dissolution may occur [101]. Though the ability of microorganisms to degrade crude oil is negatively affected in high-pH environments [102], the pH of the soil plays a major role in the degradation of crude oil by the bacteria. It has been observed that pH 7 is optimum for the significant degradation of PAHs and other *n*-alkanes (C-7 to C-25). However, bacterial strains such as *Bacillus subtilis* BL-27 showed a wide tolerance to pH values ranging from 4 to 10 during the degradation of crude oil [103].

Dubinsky et al. [104] observed that during unmitigated flow, n-alkane and cycloalkane availability were higher, which probably helped alkane-degrading bacteria flourish. According to metagenomic and meta-transcriptomic analyses carried out on a subset of samples obtained during uncontrolled flow, alkane degradation was shown to be the predominant hydrocarbon-degrading pathway expressed when *Oceanospirillaceae* and *Pseudomonas* were prevalent in the community. Numerous investigations demonstrated that oxygen loss causes biodegradation activities in soils and marine sediments to decrease drastically [105–107]. There are studies that suggest the estimated oxygen requirements for aerobic hydrocarbon degradation. As 3kg O_2 is required for every kg of petroleum contaminants, 8.6 moles of oxygen are required for every mole of diesel to be degraded [108]. Even though a variety of microbial communities typically contribute to the *in-situ* breakdown of alkane mixtures under varying soil circumstances, many soils show a similar pattern in the types of microorganisms that respond to alkane disturbance [109]. For appropriate bioremediation experiments, factors such as expense, duration, human resource, and targets must all be considered.

2.2. Classical Metabolic Pathways Involved in Degradation of Total Petroleum Hydrocarbons

Hydrocarbonoclastic bacterial species degrade PAHs due to their adaptability, vigour, diversity, and capacity to generate less hazardous metabolic intermediates. Microbes use the energy from the breakdown of hydrocarbons ranging from simple alkanes to PAHs [110]. Oxygen is necessary for the ring hydroxylation, ring cleavage, and final electron uptake in the aerobic breakdown of PAHs by bacteria. However, reductive processes are the basis of anaerobic PAH consumption [95,111]. High-molecular-weight PAHs are harder to break down because their fused aromatic structures make them very thermodynamically stable, water-repellent, and less bioavailable. Pyrene mineralisation can take place either at the C-1 and C-2 positions or at the C-4 and C-5 positions of the aromatic ring due to the action of the dioxygenase. Following that, the ring aromatisation of the dihydrodiols and the ring cleavage dioxygenase led to the synthesis of phenanthrene dicarboxylate, which is then decarboxylated to generate carboxylate. The phenanthrene dicarboxylate is eventually converted into phenanthrene carboxylate. At this stage, a cis-3,4-dihydroxyphenanthrene-4-carboxylate is formed by a deoxygenation process. This cis-3,4-dihydroxyphenanthrene-4-carboxylate then rearomatizes to form dihydroxyphenanthrene, which is then metabolised to yield hydroxynaphthoate. In addition, the pyrene breakdown occurs via the phthalate pathway as well [112,113]. The phthalate reaction forms several intermediates, including 3,4-dihydroxybenzoate and carboxyhydroxymuconate semialdehyde, both of which eventually feed into the Krebs cycle via the multiple reaction steps. Phenanthrene is a three-ringed compound whose degradation has been reported in both Gram-positive and Gram-negative microbes [114,115]. Most of the intermediates of phenanthrene degradation are shared with the pyrene degradation pathway.

Naphthalene, toluene, and anthracene have a comparatively low molecular weight and constitute most petroleum hydrocarbons [116]. Ring hydroxylating genes, such as *nagAc*, *phnAc*, *nahAc*, *nidA*, and *pdoA*, are target genes for naphthalene breakdown. In the first stage, an oxidoreductase inserts two oxygen atoms into the naphthalene rings to

generate dihydronaphthalene. The following step involves the dehydrogenase enzyme, followed by ring cleavage and oxidation [117]. The dioxygenase facilitates the cleavage of catechol and proceeds via various pathways, ultimately leading to the formation of succinyl-*CoA* and entering the TCA cycle [118]. Figure 1 represents the classical pathways for the aerobic biodegradation of various hydrocarbons, such as pyrene, anthracene, phenanthrene, and naphthalene (reconstructed from the KEGG database). Microorganisms synthesised several catabolic enzymes, such as naphthalene 1,2-dioxygenase, oxidoreductases, pyrene dioxygenase, aldehyde dehydrogenase, and many others, in every step of the degradation process. The enzymes involved in the different steps of the degradation process are also given in Figure 1.

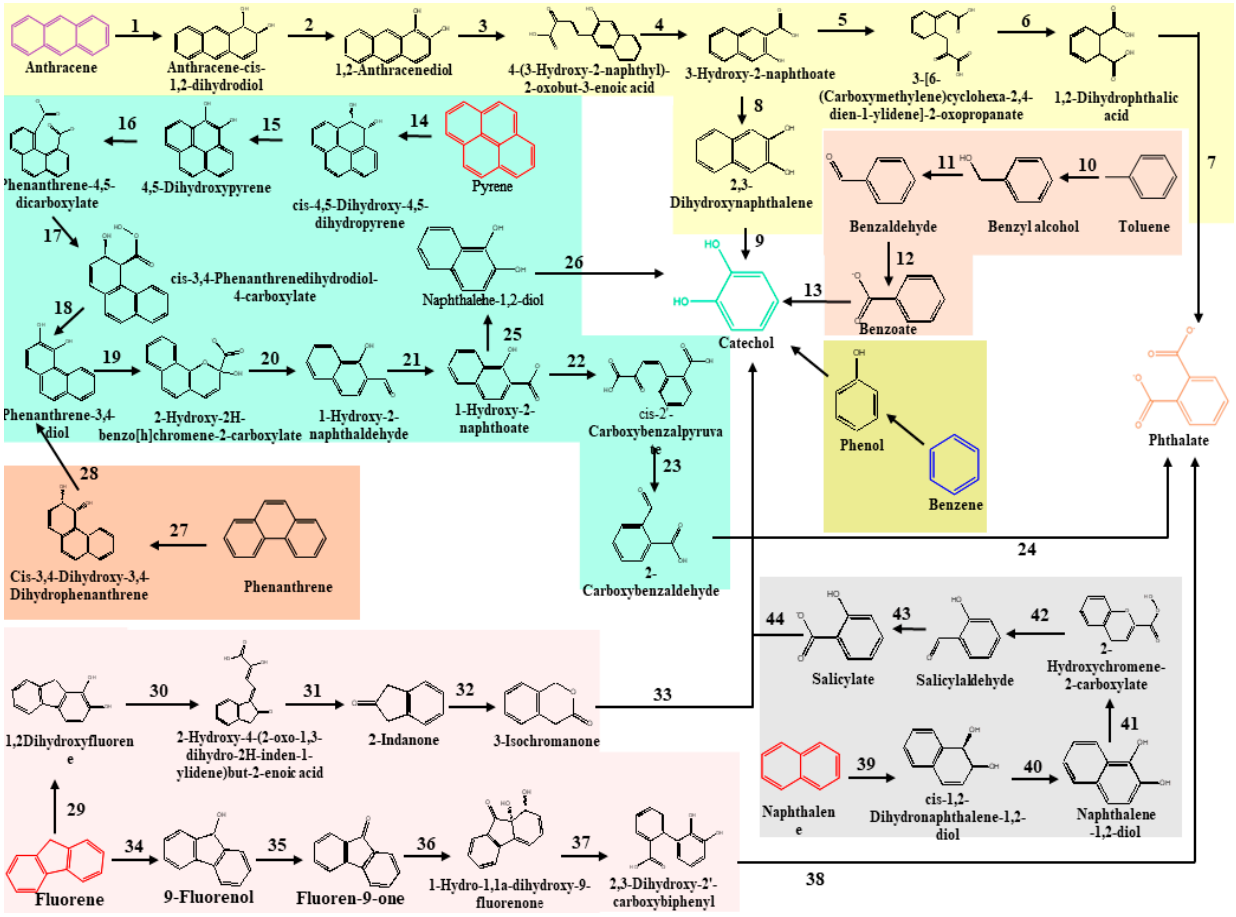

Figure 1. Pathways for degradation of anthracene, pyrene, phenanthrene, fluorene, toluene, naphthalene, and benzene until the formation of intermediate product catechol and phthalate. The enzymes involved in the reactions are 1. naphthalene 1,2-dioxygenase; 2. cis-1,2-dihydro-1,2-dihydroxynaphthalene dehydrogenase; 3. anthra-cene-1,2-diol 1,2-dioxygenase; 4. Dioxygenase; 5. 3-hydroxy-2-naphthoate 2,3-dioxygenase; 6. Oxidoreductases; 7. Oxidoreductases; 8. 3-hydroxy-2-naphthoate 2,3-dioxygenase; 9. chloroben-zene dihydrodiol dehydrogenase 10. toluene monooxygenase system protein A; 11. aryl-alcohol dehydrogenase; 12. benzaldehyde dehydrogenase; 13. dihydroxycyclohexadiene carboxylate de-hydrogenase; 14. pyrene dioxygenase; 15. dibenzothiophene dihydrodiol dehydrogenase; 16. 4,5-dihydroxypyrene dioxygenase; 17. phenanthrene-4,5-dicarboxylate decarboxylase; 18. phe-nanthrene-4-carboxylate dioxygenase; 19. extradiol dioxygenase; 20. hydratase-aldolase; 21. al-dehyde dehydrogenase; 22. 1-hydroxy-2-naphthoate dioxygenase; 23. 4-(2-carboxyphenyl)-2-oxobut-3-enoate aldolase; 24. 2-formylbenzoate dehydrogenase; 25. salicy-late hydroxylase; 26. Nah C; 27. PAH dioxygenase; 28. cis-3,4-dihydrophenanthrene-3,4-diol de-hydrogenase; 29. Hydroxylases; 30. 1,2-dihydroxyfluorene 1,1a-dioxygenase; 31. Alcohol dehy-drogenase; 32. 2-indanone monooxygenase; 33. 34. naphthalene 1,2-dioxygenase; 35. fluoren-9-ol

dehydrogenase; 36. dibenzofuran dioxygenase; 37. 1,1a-dihydroxy-1-hydro-9-fluorenone dehydrogenase; 38. 2-hydroxy-6-oxo-6-(2′-carboxyphenyl)-hexa-2,4-dienoate hydrolase; 39. Nah Ac; 40. Nah B; 41. Nah C; 42. Nah E; 43. Nah F; 44. Salicylate hydroxylate.

The solubility of alkanes in water is low and decreases with increasing molecular weight [119]. In the case of *n*-alkanes with two or more carbon atoms, aerobic degradation often begins with the oxidation of a terminal methyl group to produce a primary alcohol, which is then oxidised to the corresponding aldehyde and transformed into a fatty acid. The conjugation of fatty acids to *CoA* is followed by *β*-oxidation to create acetyl-*CoA* [120]. Some examples of regulators studied from different families are *LuxR/MalT*, *AraC/XylS*, etc. [121,122]. Asphaltenes and resins are aromatic compounds with long chains of alkyl groups that dissolve in solvents, such as *n*-heptane and *n*-pentane [123]. These components of crude petroleum are very recalcitrant in nature, and the exact intermediates formed during the degradation process are still under study [124].

The initial activation of hydrocarbons is essential for anaerobic biodegradation, and the overall enzymatic reactions involve the addition of fumarate, catalysed by a glycyl radical enzyme to yield aromatic-substituted succinates; the methylation of unsubstituted aromatics followed by the hydroxylation of an alkyl substituent via a dehydrogenase; and direct carboxylation, which may represent previous reactions. After being produced via pathways that begin with these activation reactions and end in ring saturation, *β*-oxidation, and ring cleavage reactions, with core metabolites, such as benzoyl-*CoA*, eventually being integrated into the biomass or completely oxidised [95,125]. Figure 2 represents the aerobic and anaerobic degradation steps of asphaltenes, resins, and other aromatic components of crude oil (reconstructed from the KEGG database).

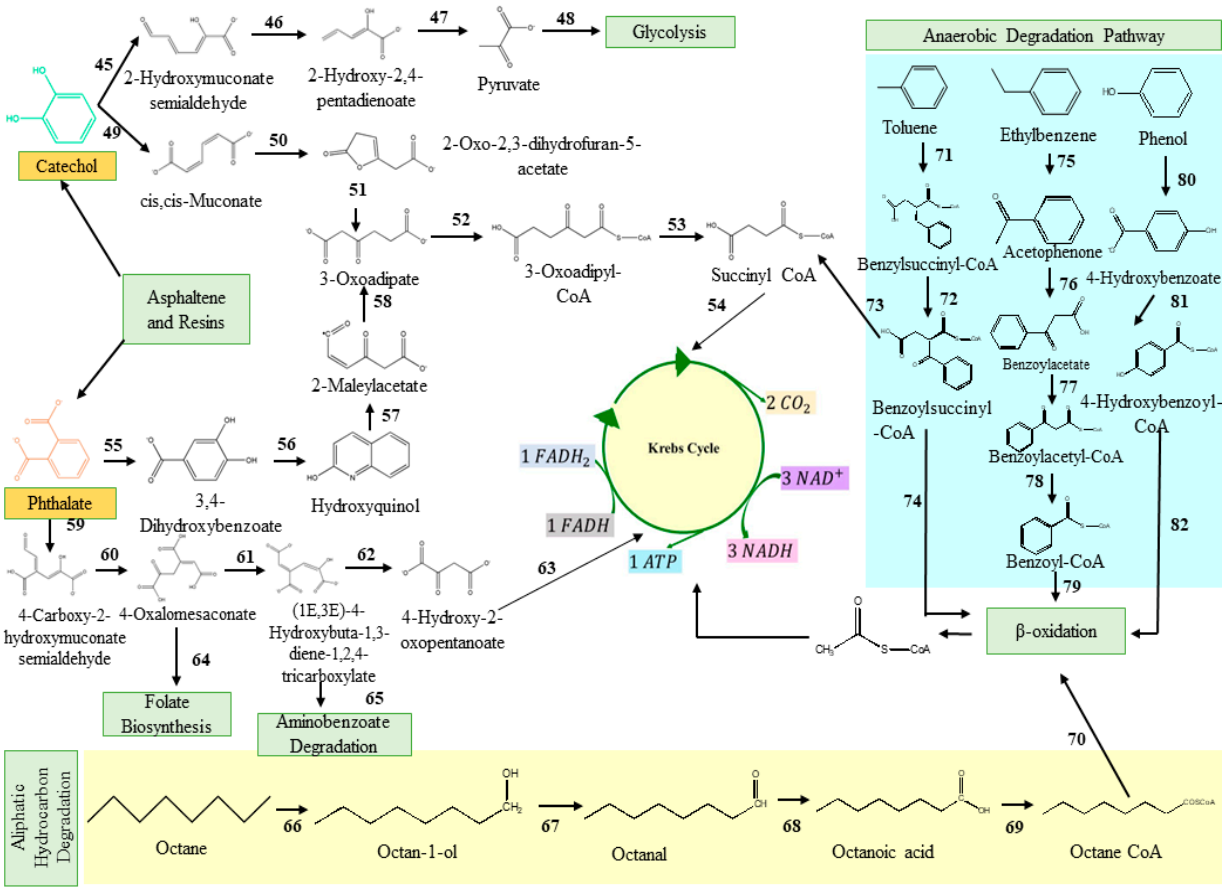

Figure 2. Terminal degradation steps of PAHs following benzoate degradation pathway via catechol and phthalate intermediates. Aliphatic hydrocarbon degradation pathway and anaerobic degradation

pathway and anaerobic degradation pathway were also given in separate colour, with both ultimately joining the common carbon metabolism pathway. The numbers representing specific enzymes viz 45. catechol 2,3-dioxygenase 46. 2-hydroxymuconate-semialdehyde hydrolase 47. 4-hydroxy 2-oxovalerate aldolase 48. Common carbon pathway's enzymes 49. catechol 1,2-dioxygenase 50. muconolactone D-isomerase; 51. 3-oxoadipate enol-lactonase; 52. 3-oxoadipate CoA-transferase; 53. acetyl-CoA acyltransferase; 54. Succnyl CoA Synthetase; 55. Monooxygenase; 56. 4-hydroxybenzoate 1-hydroxylase; 57. hy-droxyquinol 1,2-dioxygenase; 58. maleylacetate reductase; 59. protocatechuate 4,5-dioxygenase; 60. 2-pyrone-4,6-dicarboxylate lactonase; 61. 4-oxalomesaconate tautomerase; 62. 4-hydroxy-4-methyl-2-oxoglutarate aldolase; 63. Citrate synthetase; 64. Folate synthesis enzymes; 65. Aminobenzoate degradation enzymes; 66. Alkane monooxygenase; 67. Alcohol dehydrogenase; 68. Aldehyhyde dehydrogenase; 69. Synthetase 70. CoA synthetase; 71–82. Unidentified.

3. Metabolic Pathway Engineering for Bioremediation of Crude Oil Contamination

An expanding understanding of the genetics, biological pathways, and systems biology approaches of microbes offers advantages during the construction of various bioremediation strategies [126]. Several microbes degrade contaminants; however, their metabolic routes are not sufficient for the large-scale degradation of these harmful substances [127]. Baoune et al. [128] inoculated *Zea mays* with *Streptomyces* sp. H1W to remove crude oil pollution, as well as the 70% degradation of hydrocarbons (C-8 to C-30), was recorded. The bacterial consortium constructed with two salt-tolerant and biosurfactant-producing bacteria, viz., *Dietzia* sp. *CN-3* and *Acinetobacter* sp. HC8-3S, significantly degraded 95.8% of crude oil (*n*-alkanes, cycloalkanes, and aromatic hydrocarbons) in 10 days, which was more efficient than individual isolates [129]. Thus, in natural conditions, microorganisms cannot degrade crude oil contaminants. The biostimulation and bioaugmentation of biosurfactants could help in such conditions [130]. The approach of enhancing genetic and metabolic activities within cells is currently known as "metabolic engineering," which was first introduced by Bailey in 1991 as a new technical topic [131]. With microbial pathway engineering, bacterial hosts with increased enzyme activity, bioavailability of target pollutants, and production and efficiency of produced biomolecules could be introduced, and new biodegradation functionalities could also be improved. The type and concentration of contaminants also influence their degradation, which might lead to the up-and-down regulation of the various genes involved in the degradation process [132].

Although the main objectives of molecular pathway design may be the exogenous application of roadblock enzymes and the elimination of rival routes, many other crucial factors must be taken into account if the microbial isolates are to be successfully developed for the synthesis of beneficial substances [133]. The choice of a specific host, the application of specialised computational techniques, the engineering of the transcriptional regulation of enzymes, the development of novel synthetic biology techniques, and strategies to reduce metabolic strain in order to increase productivity are some of these key elements [134]. To simulate enzyme activities, identify the limitations in active bioprocessing, and gain a deeper understanding of the genetic and metabolic context of target species, metabolic scientists employ systems biology software tools and "omics" approaches [135] (Figure 3). The principles of synthetic biology and adaptive practical speciation are now frequently used in computational and scientific methodologies to overcome restrictions. The development of new tools for manipulating DNA and the improvement of technologies for sequencing genomes have made it easier to find gene clusters involved in a specific regulation. These clusters can be introduced in targeted microorganisms for the optimum production of specific metabolites [136]. Likewise, these genes or clusters can be engineered for the gain of function or loss of function via mutating specific gene targets. With the up-regulation of some pathway activators, the production of metabolites can be increased, and with the down-regulation, we can decrease the production of various pathway inhibitors [35]. Similarly, by removing one or two genes, we can restrict certain pathways to obtain the desired result. Moreover, the insertion and deletion of genes are used to discover the function and regulation of those genes [137]. In bioremediation approaches, the regulation of metabolic

pathways by providing precursor or inducer molecules is very common; these precursors might positively induce the pathway [138]. There are many approaches to achieve these strategies successfully, some of which are listed in Table 2.

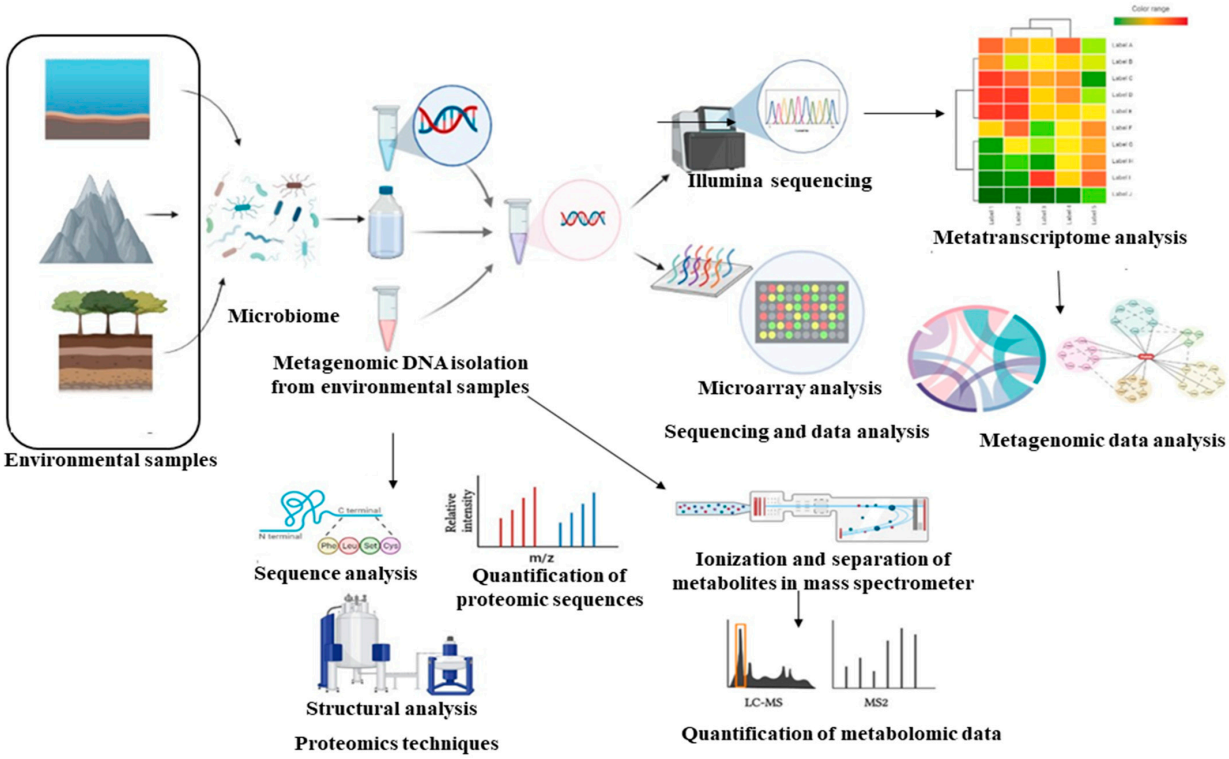

Figure 3. Illustration of sample collection from various environments and direct microbiome analysis using next-generation sequencing techniques (without the use of culture-dependent approaches). Different meta-omics approaches (metaproteomic, meta-transcriptomic, and metabolomic) were utilized to identify and detect microbial abundance and their metabolic functions in contaminated environments utilising next-generation sequencing platforms.

Table 2. Metagenomic engineering approaches using bacterial host cells and their outcomes.

Serial No.	Metabolic Engineering Technology	Types of Organisms Used	Specific Pollutants	Strategies	Result of Process	References
1	Introduction of entire gene clusters	A gene cluster from *Gordonia* sp. responsible for phthalate acid degradation (*phtBAabcdCR*) was expressed in *E. coli* BL21 (DE3)	Phthalate acid (PA) and protocatechuate acid (PCA)	Gene cluster containing complete catabolic pathways is introduced into a new host that can neutralize pollutants without inhibition.	Gene cluster encodes 3,4-phthalate dioxygenase, which totally oxidizes phthalate acid and is composed of reductase, ferredoxin, and oxygenase.	[136]
2	Engineered up-regulation of regulatory networks	*Streptomyces coelicolor*	Antibiotics	This technique entails manipulating the microorganisms so that they continually produce the activator, which further acts on specific targets.	Continuous and increased production of various secondary metabolites has been observed with continuous expression of *Streptomyces* antibiotic regulatory protein (SARP)-positive regulators.	[35]

Table 2. *Cont.*

Serial No.	Metabolic Engineering Technology	Types of Organisms Used	Specific Pollutants	Strategies	Result of Process	References
3	Engineered down-regulation of regulatory networks	*Streptomyces griseus*		Repressor or inhibitor production is interrupted in bacteria, which is the basic principle of this process.	Chromomycin synthesis rises when pathway-specific repressors are turned off.	[139]
4	Insertion and deletion of genes	*Burkholderia cenocepacia* K56–2		To obtain a desirable phenotype, it may be required to modify the route by adding or deleting one or more genes.	The modified strain can be employed in environmental bioremediation since it is simpler to genetically modify and less likely to cause severe infections.	[137]
5	Stimulation by providing precursors	*Bacillus atrophaeus* CN4	Naphthalene	Precursor or inducers have the ability to induce specific catabolic pathways involved in bioremediation.	Squamocin, a kind of acetogenin that can break down naphthalene, was a biofilm-inducing agent in the studied bacteria.	[138]
6	Gene duplication	*Pseudomonas* sp. strain ADP	Atrazine	New genetic material is created by replicating the portion of genomic DNA that includes the gene responsible for protein coding.	The *atzB* gene, which encodes the second enzyme in the atrazine catabolic pathway, was tandem duplicated in this mutant strain of *Pseudomonas* sp. strain ADP.	[140]
7	Whole-genome duplication	*Phormidium autumnale* UTEX1580	Dyes used in textile industry	Duplicating an organism's whole genome, which over time leads to speciation and divergence.	The polyploid cells of *Cyanobacterium* were observed during the process of textile dye degradation.	[141]
8	Assembly Likelihood Evaluation (ALE)	*Bacillus cereus*	Wastewater (phenolic compounds)	Effectiveness of enzymes was increased by increasing the exposure time of the microbe to the toxic pollutant.	Enhanced degradation and a significant change in cell membrane was observed after prolonged exposure to xenobiotics.	[142]
9	Heterologous expression of genes	*cph*C-I and *cph*B from *Arthrobacter chlorophenolicus*, which encodes monooxygenase complex, were expressed in *E. coli*	Chlorophenolic compounds degradation	The gene or gene cluster is cloned and expressed in other competent bacterium to increase the production of important compounds.	The inducer was produced firmly under the influence of a strong promoter, which further regulates the production of various metabolites.	[143]

3.1. Contaminant's Characteristics and Selection of Suitable Microbial Host

Hydrocarbon-based xenobiotics include agrochemicals, aromatic polycyclic hydrocarbons (PAHs), pharmaceutical substances (PhACs), personal care products (PCPs), polyphenolic compounds, disinfecting chemicals, and other industrial chemicals [69,144]. The average contamination level of total petroleum hydrocarbon (TPH) in soil was reported to be between 10^{-3} and 10^{-2} g/kg, whereas the PAHs contamination level was mostly reported to be between 10^{-6} and 10^{-3} g/kg across all continents [145]. Thus, before applying any bioremediation methods, there is a basic requirement to obtain the characteristics of the type and concentration of the pollutants [146]. There are several methods, such as scanning electron microscopy (SEM), energy-dispersive X-ray (EDX), Fourier transform infrared spectroscopy (FTIR), using a carbon–hydrogen–nitrogen (CHN) analyser,

and gas chromatography–mass spectroscopy (GC/MS) [144,147], that are used to assess contaminated and bioremediated soil.

Selecting an appropriate bacterial host is a crucial initial step in any microbial engineering system intended to create a full-fledged cell degrader [148]. Thus, developing such techniques for field applications is time-consuming, requiring many attempts to overcome the difficulties encountered. Additionally, it is challenging to maintain biotic and abiotic factors that influence and reduce degradation efficiency in natural conditions. Many reports also provide sufficient data to suggest that microbial degradation could be enhanced by the application of biosurfactants, root exudates, growth-promoting bacteria, biochar, composting, nutrients, and other biostimulation techniques. The optimisation of such techniques has been carried out over the past few years. Although indigenous bacterial stains present in crude-oil-contaminated soil cannot degrade all the components, as a result, the microbial communities present in contaminated sites synergistically degrade the contaminants in the environment. *Bacillus*, *Burkholderia*, *Sphingomonas*, and *Pseudomonas* are just a few microbial species that have demonstrated wide-range metabolism, degrading contaminants under both aerobic and anaerobic environmental conditions [149,150]. The whole-genome sequences of microbial strains, such as *Dehalococcoides mccartyi* WBC-2, *Deinococcus indicus* R1, and *Pseudomonas aeruginosa* KT2440, provide crucial information for a sustainable remediation procedure [144]. The transposon-based *lux* gene, which produces bioluminescence when coupled within a promoter for naphthalene-degrading genes, was also inserted into *P. fluorescens* HK44, along with a naphthalene catabolic plasmid called *pUTK21*. When exposed to naphthalene, the results demonstrated an increased expression of catabolic genes for naphthalene breakdown and concurrent bioluminescent activity [151].

3.2. Preference for Utilization of Substrate

Substrate utilisation by the microbial community played a significant role in metabolic pathway reconstruction. Most of the organisms use glucose as their primary source of carbon and energy. However, the microbes that can survive in sites polluted with crude oil may use toxic chemicals as their primary energy resources [152]. Based on their metabolic constitution, many species preferred using a variety of carbon sources as their primary substrate. They have a propensity to adapt to a variety nutrient-deficient environments by changing their genetic makeup [153]. SIMUP, a rational variational optimising approach, was created to identify clusters of proteins whose elimination would result in the co-utilization of substrates without prior knowledge of the transcriptional regulation [154].

3.3. Computational Application for Metabolic Pathway Prediction

The task of constructing metabolic pathways using enzymes enlisted from databases such as MetaCyc, KEGG, BRENDA, and Rhea [155], which describe the biological processes catalysed by enzymes, has now been successfully completed. The evaluation of metabolic models across different organisms is made possible by these reference-route databases, which are of tremendous significance. For recognised biochemical interactions and processes, several datasets have recently been developed, including BiGG, ModelSEED, MetRxn, UM-BBD, MOS, and Beilste Crossfile [156]. Research platforms such as ATLAS of Biochemistry [157] and METLIN [158], which contain databases of hypothetical metabolic products and processes, are very useful. The KEGG [159] and MetaCyc [160] databases catalogue a larger variety of organisms and their metabolic processes. BiGG Models (http://bigg.ucsd.edu accessed on 3 December 2022) is a centralised repository for high-quality genome-scale metabolic models and, recently, a total of 108 GEMs has been updated in the database. BRENDA provides specific enzyme features such as observed reaction kinetics [161], whereas ModelSEED allows interaction mapping between KEGG and curated GSMs [162]. To predict the optimal genetic mutation to analyse the increase in the rate and quantity of metabolite synthesis, a unique constraint-based reconstruction and analysis (COBRA) database is applied [163]. Massive quantities of "omics" data are examined by looking at species dispersion using high-efficiency route reconstruction platforms such

as MAPLE, MG-RAST, and MEGAN [164]. Several publicly available websites, such as PathPred and the University of Minnesota Pathway Prediction System (UMPPS) [165], use the method of receiving a query molecule and automatically producing the predicted chemical structure of the following compound in the anticipated pathway using computer programs. This method is also known as a "compound-filling framework." Users can choose the interest-based reactions they would like to see in this instance. PathPred is a different technique that targets both the synthesis of secondary metabolites in plants and the breakdown of xenobiotic compounds [166]. By reconstructing metabolic pathways using techniques for bioremediation, and creating enzyme-based changes in microorganisms, high-value sustainable compounds may be synthesised [167]. The use of the Biochemical Network Integrated Computational Explorer (BNICE.ch) might be advised in cases of anthropogenic pollutants with unclear or incomplete biological metabolic reactions [168]. The MetaRouter allows the presentation of all feasible routes with a substantial number of unsolved compounds via a web interface [169]. Every possible combination of enzymes (with possible homologous genes) required for converting a given substrate into its secondary intermediates or any other molecule is discovered using the database algorithm [170]. Metabolic pathway synthesis (MPS) offers important information for estimating and classifying pathways based on their physiological properties, as well as learning about their regulation and cellular activity [171].

3.4. Toxicity Determination of Metabolic Pathways

The toxicity of these anthropogenic substances may now be predicted using several in silico databases. Since pollutants negatively affect microorganisms, determining their toxicity is crucial for predicting metabolic pathways [172]. Utilising quantitative structure–activity relationships (QSARs) is an *in-silico* approach for quantifying structural correlations using empirical data [173]. Super-Toxic, a repository with 60,000 potentially toxic compounds, was developed by the biology and bioinformatics department at Charité University, Berlin, Germany [174]. ACToR is an open-access resource on toxicants that can be employed to identify possible chemical risks to the environment and public health [175]. Another web server used to predict the cytotoxicity of anthropogenic compounds in host cells is EcoliTox [176]. To develop native or recombinant biodegradation processes and leverage their expression in specific microbial hosts, in silico platforms may be incorporated into a computational platform. To avoid choosing enzymes whose enzymatic activity may be damaged by compounds in a host cell, these platforms can also be used to forecast synthesis routes.

3.5. Transcriptional Modification of Regulatory Factors

Recently, several attempts have been made to construct genetically altered microbes (GEMs) to enhance bioremediation [177]. Researchers may alter an organism's genetic makeup using molecular tools such as DNA engineering to elevate expression levels and improve enzyme activity in various circumstances. The modification of the enzyme cytochrome *P450* to enhance the binding site of degrading haloalkane dehydrogenase is the paradigmatic example of site-directed mutagenesis [178]. The main components of the recombinant DNA technology approach for bioremediation include either the development of a single microbe in which preferred biodegradation genes or routes from various organisms are combined to carry out specific interactions, or the site-directed mutagenesis-based construction of metabolites with desirable characteristics [179]. The naturally poor degradation capability of wild *Pseudomonas putida* CYP101 has been enhanced via the reconstruction of *CYP* metabolites in corresponding pathways. The mutant enzymes *Y96A*, *Y96F*, *F87A/Y96A*, and *F87L/Y96F* were three times as potent for PAHs compounds when targeted alterations were made to the active site of the CYP101 strain [164].

Carmichael and Pfaender [180] investigated the pyrene and phenanthrene degradation processes, as well as the population dynamics, of microorganisms that break down PAHs in soils with varying backgrounds of PAHs contamination. The addition of supplements

showed an enhancement in the populations of mostly heterotrophic bacteria, which readily absorbed the supplements to redirect their cellular function or activate the specific catabolic pathway used for the biodegradation of pollutants. The biphenyl fraction in polychlorinated biphenyls, which acts as an activator for the PCB-catabolic system, was added to *Alcaligenes xylosoxidans* and *Pseudomonas stutzeri*, which significantly increased their degradation capacity of PCBs [181]. However, the synthesis intensity of encoded biomolecules is usually insufficient to completely bioremediate pollutants under natural conditions [162]. Thus, the alternative integration of gene clusters in the metabolic system could overcome this problem. For example, the phthalate acid catabolic gene cluster (*phtBAabcdCR*) isolated from *Gordonia* sp. was isolated and cloned into *E. coli* BL21 (DE3) to increase degradation efficiency. Using the biphenyl-degrading and co-metabolising *Comamonas testosteroni* strain VP44 as a template, the genes encoding the ortho- and para-dichlorination of chlorobenzoates were cloned and expressed, resulting in derivatives that can grow and completely degrade chlorinate 2- and 4-chlorobiphenyl [182]. The reconstruction of the *lasI/rhlI* and *QS* genes of *Pseudomonas aeruginosa* N6P6 led to an increase in the biodegradation of pyrene and phenanthrene [183]. Inducible transcriptional expression is necessary for both natural and artificial microbial systems. The Pm from the TOL plasmid is related to the promoter genes associated with the cleavage of toluene and naphthalene. However, the *XylS*-regulating gene must be transcriptionally activated to degrade toluene [184]. Basic genetic engineering techniques, such as DNA editing and CRISPR/Cas9, are illustrated in Figure 4, showing the insertion or deletion of particular genes for target-specific degradation and substrate production. However, Figure 5 represents an example of genetic editing carried out with *Escherichia coli* for the degradation of 4-fluorophenol. Four genes, namely *fpdA2*, *fpdB*, *fpdC*, and *fpdD*, from *Arthrobacter* sp. *strain* IF1 were reconstructed into *Escherichia coli* via a multi-monocistronic vector to produce recombinant bacteria, viz., BL-*fpd*, which could efficiently degrade 4-fluorophenol. 4-FP monooxygenase (*FpdA2*), flavin reductase (*FpdB*), hydroxyquinol dioxygenase (*FpdC*), and maleylacetate reductase (*FpdD*) were the enzymes involved in the complete metabolism of 4-fluorophenol [185].

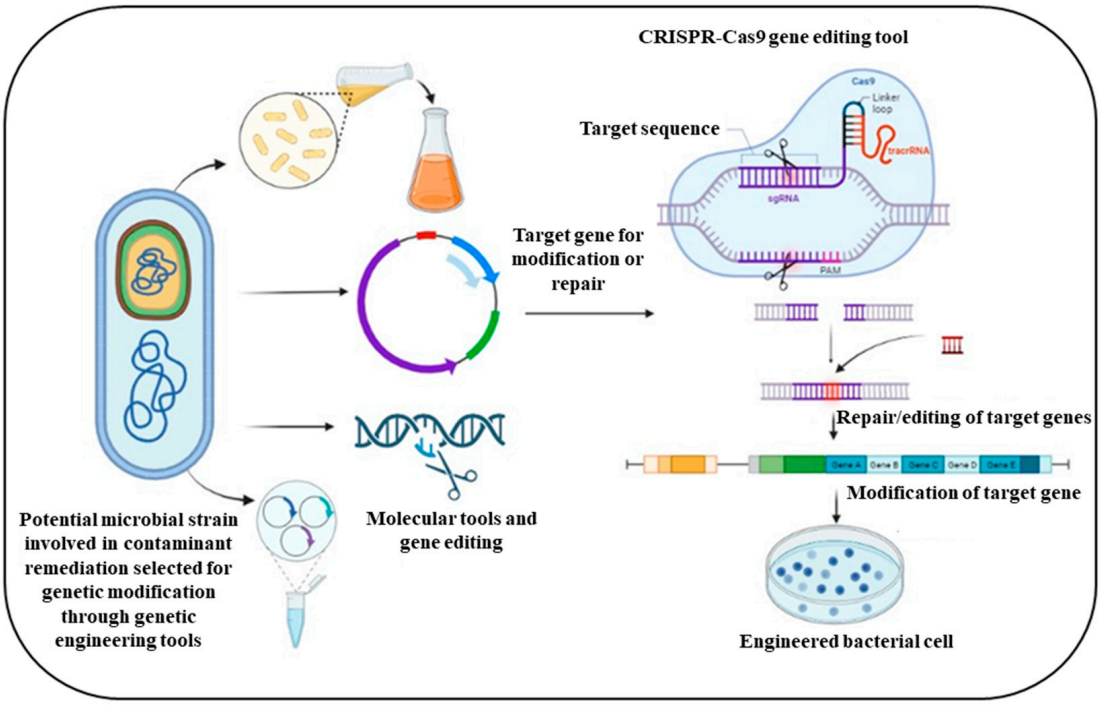

Figure 4. Illustration of genetic editing tools employed to modify and reconstruct target genes for metabolic pathway engineering in specific bacterial species. The microbes with high degradation efficiency are selected, and through cloning and other gene editing tools (TALEN, ZFNs, and CRISPR-Cas9), the efficiency of hydrocarbon degradation and its bioprocessing may be accelerated.

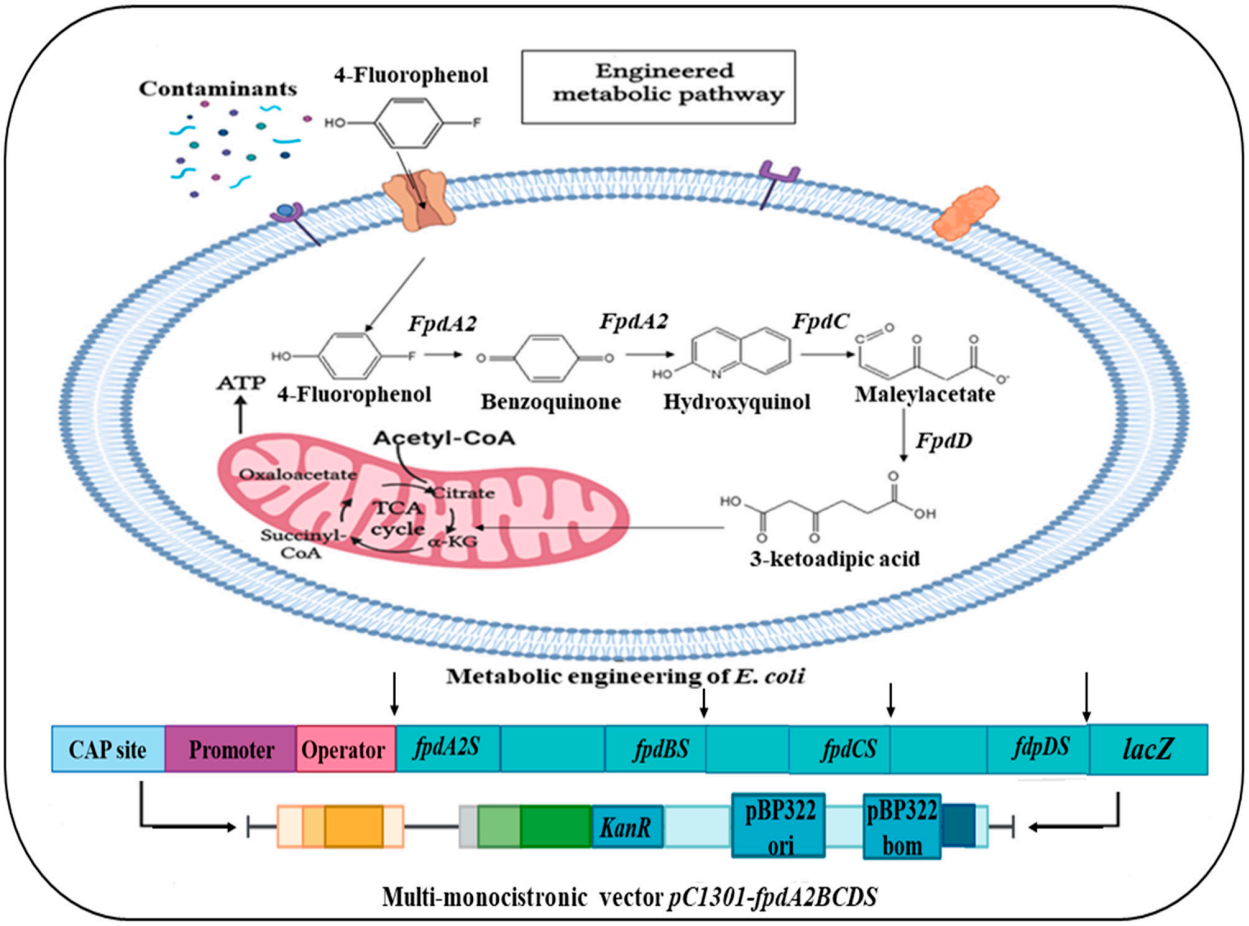

Figure 5. An illustration of metabolic engineering carried out in *E. coli* strain to enhance the degradation of 4-fluorophenol. Here, four genes from *Arthrobacter* sp. IF1 (*fpdA2*, *fpdB*, *fpdC*, and *fpdD*) were synthesized, validated, and then integrated via a multi-monocistronic carrier to generate transgenic *E. coli* strain [185].

3.6. Prediction and Engineering of Metabolic Building Blocks

The EAWAGBBD/PPS, or enviPath model, predicts biodegradation tracks using existing biocatalytic parameters [186]. Metabolic modelling may involve both metabolic flux analysis (MFA) and flux balance analysis (FBA), the two most common techniques for estimating internal fluxes [187]. Computational programs, such as Cobra 2.0, Matlab, OptKnock, or k-OptForce, can be employed to knock out or up- or down-regulate gene expression to enhance the output of by-products [188–190]. The analytical pathway tool COPASI is used to develop kinetics, discrepancy calculations, and gene stimulation times in various circumstances [191]. ChemSpider is a highly recommended source for information on the observed or anticipated physicochemical characteristics of compounds [192]. Access to more than 59 million items from 487 different data sources is available through this free repository, which was established in 2007. Regardless of the microbe of origin, the Selected Reactions Monitoring System allows for the fast, consecutive statistical analysis of the numerous proteins present in the cell [193].

4. Metabolic Engineering Studies Integrating Systems Biology

Systems biology is often used to investigate intricate molecular interactions among various biological systems. The optimisation of the bioremediation process via microbial systems biology is a novel and promising approach with great potential [194]. Systems biology, including metagenomics, proteome analysis, microarrays, fluxomic, metaproteomic, and metabolomic analyses, aids in understanding genetic regulation, identifying

target proteins, determining post-translational modifications, other metabolic cascades, and also the pathways of the signal transduction involved in bioremediation [195,196]. These "omics" approaches have provided insight into the intricate biotransformation of metabolic systems. For bioremediation to be successful, it is crucial to understand how cells work, how bacterial communities are constructed, and how metabolic processes function while under stress from harmful compounds that interfere with the normal behaviour of the microbial population.

4.1. Multi-Omics Approaches for Metabolic Pathway Engineering

Environmentalists consider metagenomics to be the first rung on the bioremediation ladder. Metagenomics is a potential tool for identifying significant microbial communities directly from environmental samples [197], with the ability to break down heavy metals, petroleum, and other hydrocarbons. Genomics studies provide a comprehensive view of the genetic material expressed in microorganisms during their exposure to contaminants. The genome sequencing of *Pseudomonas* sp. KT2440 revealed the presence of enzymes involved in the breakdown of crude oil and industrial effluents, including dehydrogenases, oxygenases, cytochromes, glutathione-S transferases, and efflux pumps [198]. Another bacterium, *Franconibacter pulveris* DJ34, isolated from crude-oil-contaminated soil (Assam, India), exhibited a diverse set of metabolic genes for the utilisation of petroleum hydrocarbons, as well as genes involved in various processes, such as dissimilatory nitrate, nitrite, and sulphite reductions; chemotaxis and biosurfactant production; and metal transportation and tolerance [199]. According to the whole-genome sequencing of the species, *Arthrobacter* strains LS16 and YC-RL1 showed metabolic networks that are engaged in the bioremediation of heterocyclic chemicals, such as naphthalene, fluorene, 4-nitrophenol, biphenyl, and xylene [200]. Similarly, the bioremediation potential of novel halophilic species, such as *Halomonas desertis* G11, which can utilise crude oil as a carbon and energy source with an inherent tolerance to salt stress, releasing a glycolipid biosurfactant, has been reported [201]. A metagenome analysis of the Black Sea's anoxic deep floor showed a cluster of catabolic genes involved in anaerobic benzoate and hydroxybenzoate degradation [202]. This strategy was also applied to evaluate aerobic microbial populations in the soil for PAH degradation. A metagenomic investigation of soil and water from beaches in the Gulf of Mexico impacted by the Deepwater Horizon oil spill revealed remarkable increases in the abundances of disease-causing *Vibrio cholera* and *Rickettsia* sp., as well as a decrease in *Synechococcus* sp. [203].

The metaproteomic approach to proteins based on environmental samples accurately describes the functional interactions among microbial communities in a specific environment [204]. According to Gillan et al. [205], the genetic diversity of the bacteria living in heavy-metal-contaminated locations is evident in their production of exopolymers and enzymes. A metaproteome study of *Mycobacterium vanbaalenii* PYR-1 grown in the presence of HMW-PAHs suggested the production of enzymes, such as monooxygenases, dioxygenases, naphthalene-inducible dioxygenases, catalase, peroxidases, and dehydrogenases [206]. Similarly, some *Clostridiale* bacterial species express *bamC*, *bamD*, *bzlA*, and *ubiD* genes when exposed to benzene [207]. *P. putida* was cultivated on benzoate-containing media, which showed that the catabolic enzymes *CatB*, *PcaI*, and *PcaF*, as well as *DmpC*, *D*, *E*, *F*, and *G*, were engaged in the ortho-cleavage and meta-cleavage pathways [208]. Furthermore, metaproteomic studies were also used to investigate the adaptive response of *Exiguobacterium* sp. S17, which was isolated from an Andean Lake stromatolite, for arsenic-induced stress [209].

The central objective of transcriptomic studies is to investigate the up-regulation and down-regulation of genes in response to environmental contaminants [210]. Using DNA microarray technology, cis-dichloroethane (cDCE) was incorporated into the *Polaromonas* sp. JS666 strain, and transcriptome analysis was carried out to identify the genes that were elevated in the presence of cDCE [211]. Transcriptome studies of three *Pseudomonas aeruginosa* strains, N002, TP16, and J001, showed a total of 81, 269, and 137 significant

DEGs in the presence of crude oil contamination [212]. Furthermore, a transcriptomic analysis of the expression profile of the bacteria *Pseudomonas extremaustralis* in aerobic and microaerophilic conditions was carried out [213]. It showed that in the presence of hydrocarbon contamination, alkane-degrading genes, such as *alkB*, were overexpressed. The process of crude oil degradation at low temperatures has been shown by a transcriptomics investigation of *Pedobacter steynii* DX4 [164]. The transcriptome analysis of the hydrocarbon-degrading *Achromobacter* sp. HZ01A, grown in crude oil contamination, showed that certain genes were active for degradation activities, whereas other genes involved in cell motility, metabolism, and protein-coding were down-regulated [214].

Another thriving and quickly developing area of systems biology at the intersection of biological sciences and chemistry is metabolomics, which analyses the full spectrum of cellular metabolites generated by microbial organisms [215]. Metabolomics covers all the components of nucleotides and proteins as well as a wide range of organic metabolites with various functional groups. Additionally, microbial metabolites are important in both intra- and inter-species microbial interactions. For example, it is difficult to degrade vinyl chloride (VC) since it has a cancer-causing trait. The metabolomic study identified the microorganisms responsible for VC degradation, which included *Actinobacteria*, *Proteobacteria*, and *Bacteroide* [216].

To examine cellular metabolites generated under certain environmental conditions, metabolic flux analysis, also known as fluxomics, monitors the rate of metabolic processes in real-time [217]. It is a useful technique for examining routes and figuring out the fate of metabolites. A high-throughput genotypic investigation of the phenotype's expression in its environment is called phenomics [218]. Phenomics primarily includes the characterisation of a specific group of traits, which necessitates the mapping of the genetic material. Similarly, ionomics studies integrated with genomics can efficiently quantify and identify elemental compound composition to discover relevant gene mutations and investigate stress resistance in the microbiome [219]. Figure 3 represents the different omics techniques, providing a culture-independent platform for the direct isolation of microbial DNA, RNA, and proteins from environmental samples. Further, according to the required information, the samples are processed via metagenomics, proteomics, and transcriptomics on different NGS platforms, and the raw sequences are then analysed using various computational databases and tools.

4.2. Computational Analytical Software Used in System Biology

The use of omics approaches in systems biology generates a huge quantity of data, and it is thus crucial to integrate and interpret these complicated datasets. In silico approaches are employed to analyse genes, proteins, and the functioning of cells, and to assist in building modern enzyme-based bioremediation processes [220]. Integrated Microbial Genome and Microbiome (IMG/M) is a platform for investigating the functional potential of microbial populations and is one of the online tools available for metagenome studies [221]. A comparison study of the genome and metagenome sequences available on the Joint Genome Institute (JGI), Integrated Microbial Genomes and Microbiomes, and MetaBioME was conducted based on the metagenome sequences [222,223]. Orphelia is a tool for finding Open Reading Frames (ORFs) in short metagenomic sequences of uncertain phylogeny [224], while MetaBioME is a resource focused on new enzyme discovery from metagenomic data. Another free online integrative framework resource is MetaRuter, which represents data on numerous biochemical substances, enzymes, interactions, and organisms associated with biodegradation and bioremediation processes [225]. The most frequently employed approaches for the stoichiometric study of metabolic networks are flux balance analysis (FBA) [226] and metabolic flux analysis (MFA) [227]. Platforms such as OpenMS [228], MetaboloAnalyst [229], and MetaboLights [230] provide open access to metabolomics data. OptReg enables the *in-silico* regulation and alteration (positive or negative) of the enzymes and metabolic pathways involved in the process of effective bioremediation [33].

4.3. Limitations in System Biology Approach

Systems biology investigations are usually performed by a diverse team of researchers capable of developing the required technologies and computational tools. Effective and capable computational tools are necessary to manage the vast amount of data [231]. Moreover, there is a substantial challenge in calibrating computational tools, models, and algorithms to an appropriate level. Another limiting factor is the high costs associated with processing samples and the need for specialised equipment. These issues have limited the application of systems biology for bioremediation thus far. In bioremediation, low molecular weight intermediates are equally important, and integrating omics methods, particularly metabolomics, is extremely difficult [232]. Systems-based methods mainly rely on data from open databases. However, the datasets are frequently unfinished, unstandardised, or improperly annotated.

5. Synthetic Biology Approach in the Field of Metabolic Engineering

Synthetic biology is an approach that deals with developing and reconstructing metabolically functional species with certain fixed attributes. Genomic networks and biochemical functions may be designed and modified using computational simulations and engineering tools. Vectors, also known as chassis, are used to introduce changes in the appropriate host [233]. The top-down strategy, which creates artificial species by changing the genetic makeup or metabolic pathways, and the bottom-up method, which changes organisms using basic molecular building blocks, are two important techniques for building the right chassis [234]. Techniques based on synthetic biology include the development of biosensors, the design of genetically modified microbes, and the use of microbial consortiums to provide novel tools for improving the outcome of the degradation process [235].

5.1. Construction of Synthetic Consortia to Enhance Biodegradation

A combination of more than two microbial species that exhibit symbiotic interaction is defined as a "microbial consortium". Due to the synergistic linkages established by the complementing functions and metabolic capabilities of each species, a consortium often performs more effectively than a single microbe [236]. Some of the key attractions for developing different synthetic biology approaches include the addition of exogenous molecules, which regulate cell interactions and gene expression; regulating organism-to-organism syntrophic interactions; and modulating microbial consonant intercellular signalling [217]. The consortium of two biosurfactant-producing *Bacillus* strains and hydrocarbon-degrading strains showed a reduction of 84% for crude oil in FTIR and GC/MS studies after five weeks of experimentation. The degradation of hydrocarbon plastic waste can be significantly improved by using indigenous or modified bacterial consortiums [237]. Another report describes using a synthetic anaerobic bacterial consortium of *Desulfatiglans parachlorophenolica* DS, *Dehalobacter* sp. FTH1, and *Clostridium* sp. Ma13 for the complete degradation of 2,4,6-tribromophenol [238]. Compared with the wild type, the engineered consortium of the *P. aeruginosa* PH1 strain can degrade about 71% of phenanthrene within nine conjugative days [239].

5.2. Risk Assessment of Synthetic Consortium

One of the major issues pertaining to the environmental application of metabolically engineered cells is the concerns for genetic alterations. The risk analysis of genetically engineered microorganisms (GEMs) is crucial because they may have hazardous and pathogenic effects in the environment that might transfer to other nearby cells. Thus, two key strategies that might reduce these negative effects are using non-antibiotic marker screening and limiting genetic recombination to native species. Approaches for biocontainment involving the cellular, transcriptional, or auxotrophy systems have been explored to address this issue. Cross-feeding has certain drawbacks, even though it is frequently employed to build synthetic microbial communities [240]. For example, the marine *Prochlorococcus* SAR11 provides carbon sources to other bacteria but struggles with sulphur resources [241]. Therefore,

it is imperative to carry out further in situ studies to examine the behaviour of GEMs for the application of hydrocarbon remediation in an unconstrained environment, as well as their appropriate biomonitoring, in order to validate this option in bioremediation as one of the most effective and reliable.

5.3. Synthetic Orthogonal Approach for Bioremediation Enhancement

The major objective of any orthogonal systems biology approach is the determination of organismal traits ranging from single molecules to entire cells without interacting with a pre-existing biological framework [242]. However, in a natural environment, full orthogonalisation cannot be achieved. The use of non-natural genetic codons, alternative transcriptional machinery, synthetic toggle switches, and modifications to biological circuits are some of the most conspicuous examples of orthogonalisation [243]. These techniques are used to change the specificity of codons or enhance the dominance of genetically predetermined amino acids for the synthesis of non-natural samples. These prompted the engineering of a metabolic and signalling pathway by the integration of a synthetic amino acid codon into the protein's non-sense sequence [244]. Thus, the synthesis of an entirely fresh set of molecules may include a new functional route. These modified circuits barely interact with the cells' exhibiting counterpart routes to reduce the possibility of cross-inhibition [245]. This strategy may enhance the sensitivity, reliability, and functionality of a single organism or microbial consortium for the construction of potential complete-cell biosensors and degraders of widespread chemical substances.

Isaacs et al. [246] demonstrated that replacing every 314 of the TAG stop codons in the *E. coli* genome with homologous TAA codons provided massive substitutions of higher-order variants despite having a fatal outcome. Wang et al. [247] developed two biosensor-based *E. coli* strains with a signal transduction modification in the *hrpR* and *hrpS* genes along with the *HrpL*-promoter element from *P. syringae*. Similarly, cyclic di-GMP is the main regulator of the complex network, directing the transition between cells and biofilm formation. This trait was used to develop an orthogonal genomic tool to change the biochemistry of *P. putida* [248]. The cyclohexanone-responsive expression method was used to precisely control the diguanylate-cyclase-encoding *yedQ* gene in *E. coli.* Additionally, a biofilm-forming *P. putida* strain was modified, and a synthetic operon for encoding the enzymes needed for 1-chlorobutane biodegradation was introduced. When turned on, this showed an activation of dehalogenase activity [249]. Microbial fuel cells (MFCs), which may generate electrical impulses during the breakdown of organic contaminants; the continuous biomonitoring of p-nitrophenol in industrial wastewater; and the degradation of atrazine and copper from mine effluent have all been shown using biosensors with an output voltage from *Geobacter sulfurreducens* and *Shewanella oneidensis* MFCs [250].

5.4. Experimental Strategies for Metabolic Pathway Engineering

Experimental approaches for metabolic pathway engineering include genome editing, the expression of heterogenous genes, and adaptive laboratory evolution. CRISPR-*cas*, ZFN, and TALEN are the primary gene editing technologies. CRISPR is a 30–40 bp direct repeat sequence that complements the foreign sequence and is separated by a spacer region [251]. After processing and transcription, crRNA is created. By inducing a double-strand break (DSB) at the designated target, CRISPR/Cas9 may be used to alter (delete or insert) the target gene from the genome. Because of its greater level of compatibility with archaeal and bacterial networks, CRISPR-*cas* is an appropriate tool that could be used in a variety of bioremediation studies opening up the possibility of the creation of more complex, programmable, and effective gene networks [252]. The zinc finger nucleases (ZFNs) are the most widely used synthetic restriction enzymes. The zinc finger proteins (ZFPs) present in zinc finger nucleases are eukaryotic transcriptional factors with a specific DNA-binding domain [253]. This is an effective gene editing technique with gene knock-out and knock-in functionality for prokaryotic and eukaryotic gene systems [254]. Transcription activator-like effector nucleases are referred to as TALENs, and are a cutting-edge tool for genetic

manipulation and modification. With TALENs, the editing of the target gene or gene of interest is currently recommended [181]. The TALENs are a powerful gene editing technique because they have two TAL protein domains, one for sequence fragmentation and the other for identifying and binding the incredibly precise site [255]. *Pseudomonas putida* KT2440 grown in trinitrotoluene for the induction of *pnrA*, *xenD*, and *acpD* detoxifies genes. Furthermore, the *Comamonas testosteroni* strain VP44 was able to grow on and entirely dechlorinate 2- and 4-chlorobiphenyl by being cloned and expressing the genes that encode enzymes for ortho- and para-dechlorination [143].

Further, the bioremediation of anthropogenic substances could be enhanced by the improvement in the efficiency of already-existing natural pathways [256]. For instance, in a batch procedure carried out in the laboratory, *Bacillus cereus* was cultivated in an environment of phenol. The cell membrane of the bacterium underwent considerable modifications that accelerated the biodegradation of phenol in comparison with the original strain [257]. Recently, *Burkholderia* sp. SZL-1-mutant libraries were created by knocking out the triazophos hydrolase gene (*trhA*) and screening the efficient isolates with the best capacity to degrade triazophos substances [258].

6. Conclusions and Future Perspective

The need for the metabolic engineering of hydrocarbon degradation pathways must be realised with the prospect of the improved and efficient bioremediation of polluted environments. Crude oil contamination is a growing concern due to its negative effect on the environment and its harm to all living organisms. Though there are several bioremediation strategies available to deal with the challenges of removing recalcitrant crude oil contaminants from the ecosystem, bioaugmentation with a few bacteria or a single bioremediation pathway is not enough to degrade all the components at the same time. Additionally, in the natural environment, several biotic and abiotic factors, such as temperature, snow, rainfall, soil pH, and low nutrient content, influence the bioremediation process. Furthermore, the availability of multi-omics-based tools, such as genomics, proteomics, transcriptomics, metabolomics, etc. provides significant information and generates sufficient data for a better understanding of microbial behaviour in xenobiotic-contaminated environmental conditions for efficient bioremediation. Thus, these may be exploited as platforms for metabolic-pathway-engineering tools that may provide excellent strategies for significant biodegradations in natural ecosystems. Although genetic engineering approaches have the ability to improve catabolic genes and related pathways, due to the chances of horizontal gene transfer among bacterial communities, the application of genetically modified organisms in the environment becomes challenging for researchers. A better understanding of metabolic processes using gene editing tools and a systems biology approach can overcome such in situ bioremediation applications. Metabolic engineering involves the utilisation of the already-established pathways to accelerate and enhance bacterial degradation for the restoration of contaminated environments. Therefore, the advantageous gene editing technologies TALEN, ZFNs, and CRISPR/Cas9 could be used to improve the function of certain microbes with specific genes and enzymes involved in xenobiotic remediation. On the other hand, utilising synthetic ecosystems or cells would be beneficial in altering their behaviour and functions, or creating entirely new functions that are not found in nature, thus leading to advancements. Despite tremendous advances in the creation of various in silico databases, programs, and computational models for the investigation of microbial activities, the main challenges are in applying appropriate, approachable, and simple bioinformatics tools to analyse output findings and develop relevant conclusions. Thus, researchers from various fields must cooperate to overcome such difficulties by sharing data, creating more publicly available database platforms, and supporting innovative metabolic engineering approaches with the purpose of the effective and efficient biodegradation of crude oil contaminations from different environments. However, concerns for the application of genetically modified organisms in the environment will be a challenge

and should be addressed in parallel with the metabolic engineering approach in order to develop a holistic strategy.

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Abbreviations

GMO	Genetically modified organisms
GEMs	Genetically engineered microorganisms
HMW	High molecular weight
LMW	Low molecular weight
PAH	Polycyclic aromatic hydrocarbons
NSO	Nitrogen, sulphur, and oxygen
DEGs	Differentially expressed genes

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