JBródka PUBLISHER

Review Article

# CRISPR genetic engineering for enhanced bioremediation potential of rhizobacteria

**Stanley Chukwudozie Onuoha [1,*], M. D. Damian [2]**

[1] Department of Biotechnology, Faculty of Science, Ebonyi State University, P. M. B. 053, Abakaliki, Nigeria
[2] National Biotechnology Development Agency, Nigeria
*Correspondence: onuoha.stanley@ebsu.edu.ng (SCO)

**ABSTRACT**
CRISPR-based genetic engineering provides a revolutionary method for improving the bioremediation potential of rhizobacteria. Species such as *Bacillus subtilis*, *Pseudomonas fluorescens*, and *Azospirillum brasilense* exhibit inherent efficacy in pollution breakdown and the enhancement of plant development. Utilising CRISPR-Cas systems like Cas9, CRISPRi, and CRISPRa, these microorganisms can be modified to enhance stress resilience, enzymatic function, and pollutant specificity. The method encompasses a framework that includes strain selection, whole-genome sequencing, and bioinformatic analysis to uncover essential catabolic and resistance genes. Guide RNAs are engineered to target specific loci and are associated with Cas proteins for gene editing. The resultant structures are introduced into rhizobacterial hosts via electroporation or conjugation. The efficacy of transformation and genomic integration is confirmed using PCR, gel electrophoresis, and sequencing. Modified strains are subjected to phenotypic screening to evaluate pollutant breakdown capability, enzymatic efficiency, and alterations in metabolic flux. Laboratory assays are succeeded by greenhouse trials to assess phytoremediation synergy, root colonisation efficacy, and rhizosphere stability under simulated field conditions. Environmental and biosafety factors are crucial in the implementation of CRISPR-modified rhizobacteria. This encompasses measures to reduce off-target effects, biocontainment methods including kill-switch devices, and monitoring for horizontal gene transfer by metagenomic surveillance. Ethical and regulatory compliance is achieved by following biosafety guidelines and conducting environmental risk assessments to maintain ecological stability. In conclusion, CRISPR-modified rhizobacteria constitute a promising and precise approach for environmental remediation. Their capacities for pollutant breakdown, coupled with host plant compatibility, render them effective instruments in sustainable environmental biotechnology.

**KEYWORDS:** Bioremediation; CRISPR-enhanced rhizobacteria; Heavy metals; Environmental pollution

**DOI:** http://dx.doi.org/10.5281/zenodo.21427230

Submitted: 15 November 2025
Revised: 23 February 2026
Accepted: 15 March 2026
Published: 26 March 2026

**ISSN:** 2449-8955
**Copyright:** © 2026 The Author(s). Published by JBródka Publisher. This is an open access article under the CC BY 4.0 license.
**Publisher's Note:** The opinions and statements in this publication are solely those of the Authors and do not represent JBródka Publisher or the editors, who disclaim responsibility for any consequences arising from the use of the presented information.

## INTRODUCTION

Environmental pollution has escalated globally in recent decades as a result of heightened industrial activity, agricultural practices, and urbanisation [1]. Heavy metals, petroleum hydrocarbons, and pesticides are particularly persistent pollutants that present enduring risks to ecological and human health [2]. Conventional remediation procedures, such as physical and chemical approaches, frequently demonstrate high costs, inefficacy, and environmental disruption, hence prompting the need for sustainable alternatives [3]. Bioremediation, a technology employing living organisms to decontaminate polluted areas, has surfaced as a viable alternative to traditional treatments. In this context, plant-associated beneficial microorganisms, especially plant growth-promoting rhizobacteria (PGPR), have garnered interest for their inherent capacity to colonise plant roots and improve phytoremediation [4]. PGPR not only promote plant development under stress conditions but also significantly contribute to the degradation and transformation of diverse environmental pollutants [5].

The inherent biodegradation ability of natural rhizobacteria is frequently restricted by variables including gene expression limitations, adverse environmental circumstances, or the lack of particular catabolic pathways necessary for the breakdown of complex contaminants [6]. To address these constraints and improve the efficacy of bioremediation, genetic engineering techniques are being progressively investigated. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated proteins (Cas) have transformed microbial engineering through their precision, efficiency, and versatility [7]. CRISPR-Cas technology, initially identified as a component of the bacterial immune system, facilitates precise genome editing by creating double-stranded breaks at designated sites within the DNA sequence [8]. This enables accurate insertion, deletion, or alteration of genes, providing unparalleled control over microbial metabolic pathways [9]. In bioremediation, CRISPR facilitates the enhancement or introduction of novel catabolic genes into rhizobacteria, thereby augmenting their ability to breakdown or detoxify a wide array of environmental toxins [10].

Recent research have shown that CRISPR can be efficiently employed to modify PGPR for improved degrading capabilities. By including genes associated with hydrocarbon breakdown or metal tolerance, researchers have developed modified strains that can endure and remediate severe, contaminated settings [11]. Moreover, CRISPR-based instruments can dynamically regulate gene expression, facilitating the optimisation of metabolic pathways associated with pollutant breakdown [12]. These developments indicate that CRISPR possesses significant potential to enhance rhizobacteria as bioremediation agents. The soil microbiome, especially rhizobacteria, is recognised for its genetic variety and adaptability, rendering it a prime candidate for CRISPR-based manipulation [13]. The incorporation of foreign genetic material into soil microorganisms has traditionally encountered obstacles, such as low transformation efficiency and off-target consequences [14]. The advancement of refined CRISPR variants like CRISPR-Cas12 and CRISPR-Cas13 provides researchers with systems exhibiting diminished off-target effects and enhanced targeting abilities, making them more appropriate for environmental applications. Furthermore, the integration of CRISPR with synthetic biology methodologies facilitates the creation of tailored genetic circuits that react to environmental stimuli, resulting in conditional expression of genes responsible for pollutant degradation [15]. This technique reduces the metabolic burden on host bacteria and guarantees that degradation pathways are active just in the presence of contaminants, hence improving survival and functionality in field circumstances [1]. Intelligent microbial systems are anticipated to be essential in the development of next-generation bioremediation technologies.

The utilisation of CRISPR in rhizobacterial bioremediation extends beyond merely augmenting degradation pathways. This approach can enhance plant-microbe interactions by genetically modifying bacteria to generate more effective phytohormones or to adjust plant stress responses, hence synergistically improving phytoremediation results [16]. Furthermore, engineered rhizobacteria can be designed to synthesise biosurfactants or chelating agents, enhancing the bioavailability of hydrophobic contaminants or heavy metals, hence promoting their absorption and breakdown [17]. Notwithstanding its transformative promise, the utilisation of CRISPR for bioremediation is still in its early stages, and numerous obstacles persist. Prominent among them are the hazards linked to the introduction of genetically modified organisms (GMOs) into natural ecosystems, encompassing potential horizontal gene transfer and ecological disruptions [17]. Consequently, regulatory frameworks and biosafety protocols must progress in tandem with technological improvements to guarantee appropriate utilization [18].

In light of biosafety issues, researchers are investigating methods to create "biocontained" synthetic rhizobacteria utilising CRISPR-based kill switches or dependency systems that restrict life beyond specified settings [17]. Synthetic protections are essential for the widespread acceptance and use of CRISPR-engineered microorganisms in practical applications [18]. Simultaneously, public participation and policy discussions are crucial for addressing ethical issues and fostering informed decision-making in environmental biotechnology. A burgeoning domain is the application of CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa), which provide reversible gene silencing or activation without modifying the DNA sequence [19]. This offers a non-invasive method to examine and enhance the microbial reaction to contaminants while maintaining the genetic integrity of the host strain. The capacity to precisely adjust gene expression in this way creates new opportunities for the development of environmentally resilient microbial consortia designed for specific contamination profiles [20]. Moreover, progress in metagenomics and systems biology has enabled the discovery of new genes and regulatory networks associated with pollutant breakdown, which can be utilised through CRISPR technology to develop highly efficient microbial strains [21]. These findings are essential for formulating logical, data-informed engineering solutions that optimise the repair capabilities of rhizobacteria while reducing undesired consequences. The increasing necessity to effectively tackle environmental pollution makes the combination of CRISPR technology and rhizobacterial bioremediation a pioneering research domain with significant potential. It connects microbial ecology with synthetic biology, providing a scalable, efficient, and environmentally sustainable solution to significant contemporary environmental issues [21]. As additional empirical evidence is obtained from laboratory and field studies, the framework for implementing CRISPR-engineered rhizobacteria in bioremediation will become more defined, paving the way for extensive adoption.

CRISPR genetic engineering significantly enhances the bioremediation potential of rhizobacteria by facilitating the exact alteration of genes associated with pollution breakdown, stress resistance, and plant contact [22]. This technology, through the advancement of intelligent, adaptive, and effective microbial strains, is poised to transform environmental biotechnology and facilitate the emergence of more sustainable remediation solutions [23]. The ongoing integration of CRISPR innovations with microbial ecology, bioinformatics, and systems biology will certainly enhance the resources available for tackling global pollution issues.

## RHIZOBACTERIA IN BIOREMEDIATION AND CRISPR TECHNOLOGY

### RHIZOBACTERIA IN BIOREMEDIATION

#### *DEFINITION AND CHARACTERISTICS OF RHIZOBACTERIA*

Rhizobacteria are a category of soil-dwelling microorganisms that occupy the rhizosphere, the soil area directly affected by plant roots. These bacteria participate in mutualistic relationships with host plants, facilitating nutrient cycling, disease resistance, and promoting plant growth [3]. Rhizobacteria are categorised as either free-living or symbiotic and may display characteristics such as nitrogen fixation, phosphate solubilisation, and siderophore synthesis. Their metabolic diversity and capacity to adapt to various soil conditions render them very appealing for applications in environmental biotechnology [5].

#### *FUNCTION IN PHYTOREMEDIATION AND ENVIRONMENTAL DECONTAMINATION*

Plant-growth-promoting rhizobacteria (PGPR) enhance phytoremediation by augmenting plant vitality and resilience in contaminated ecosystems. They synthesise phytohormones such as indole-3-acetic acid (IAA), gibberellins, and cytokinins that facilitate root elongation and biomass accumulation [5]. Moreover, these microbes augment the bioavailability of heavy metals and organic pollutants by secreting chelators, acids, and enzymes [17]. Consequently, the plants can absorb, stabilise, or volatilise pollutants with greater efficacy (Figure 1).

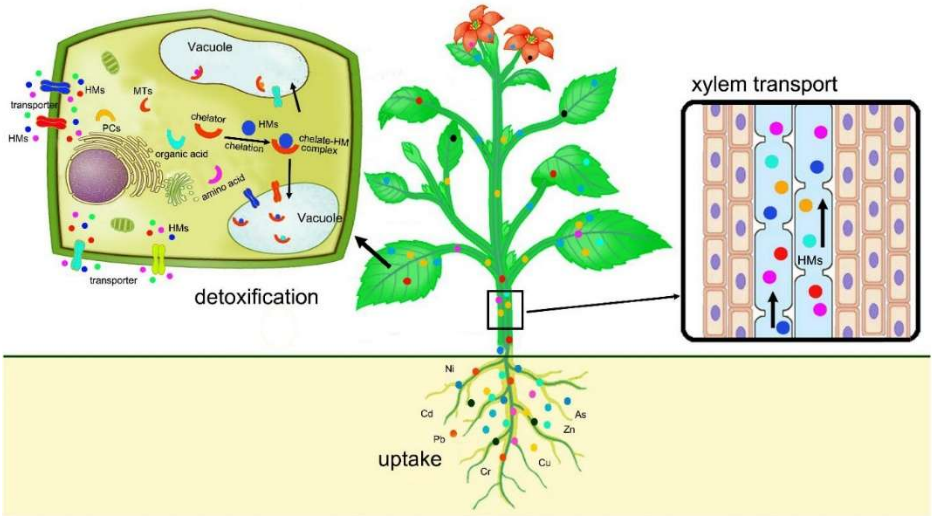


**Figure 1.** Rhizobacteria phytoremediation and environmental detoxification [17].

Rhizobacteria play a crucial role in the enzymatic breakdown of xenobiotics. Enzymes like dehydrogenases, laccases, and monooxygenases facilitate the degradation of intricate organic contaminants such as petroleum hydrocarbons, pesticides, and phenols [17]. Microbial activity is additionally affected by elements such as soil pH, temperature, moisture, and the availability of co-substrates. The collaboration between rhizobacteria and plants enhances phytoremediation efficacy while maintaining soil health, fertility, and structure in contaminated areas.

The bioremediation potential of PGPR is particularly apparent in hydrocarbon-contaminated soils, where they promote rhizodegradation by improving root exudate compositions and microbial metabolic functions. Research indicates that plants inoculated with PGPR cultivated in diesel-contaminated soils demonstrate enhanced root and shoot biomass, suggesting a significant relationship between microbial interaction and pollution tolerance. Microbial consortia can be customised to address certain contaminants, providing targeted remediation options. Besides improving pollutant absorption, rhizobacteria can stimulate systemic resistance in plants, safeguarding them from oxidative stress and pathogen assaults frequently intensified by toxic surroundings. The dual role of PGPR as bioremediators and bio-protectors establishes them as essential bio-agents in sustainable soil management and ecological restoration. Progress in microbial ecology and metagenomics consistently reveals novel strains and metabolic pathways that enhance the capabilities of rhizobacteria in extensive environmental detoxification.

### *MECHANISMS OF HEAVY METAL TOLERANCE AND REMEDIATION BY RHIZOBACTERIA*

Heavy metals represent a considerable environmental hazard because to their non-biodegradable and poisonous characteristics. Rhizobacteria mitigate heavy metal stress by many processes such as biosorption, bioaccumulation, precipitation, and enzymatic detoxification [3]. *Bacillus* and *Pseudomonas* strains are recognised for producing extracellular polymeric substances (EPS) that sequester metal ions, therefore diminishing their bioavailability.

Siderophore-mediated chelation is a further mechanism by which rhizobacteria sequester metals like iron, cadmium, and lead. Moreover, metal resistance genes that encode efflux pumps, metal-binding proteins, and redox enzymes enable bacteria to endure and operate in contaminated soils [3]. These genes frequently exist on mobile genetic components such as plasmids and transposons, facilitating horizontal gene transfer and adaptation within microbial communities.

Furthermore, the synthesis of metallothioneins-cysteine-rich peptides that may bind heavy metals-enables rhizobacteria to mitigate toxicity while preserving cellular homeostasis. Certain rhizobacteria can perform redox transformations, turning dangerous metal ions into less harmful forms; for instance, reducing Cr(VI) to Cr(III) or U(VI) to U(IV). These redox processes are essential for the immobilisation and detoxification of heavy metals in situ. Bioprecipitation, the process by which metals are transformed into insoluble salts and deposited into the soil matrix, is a crucial mechanism utilised by rhizobacteria. This aids in reducing leaching and averting secondary groundwater contamination. The interplay between rhizobacteria and heavy metals demonstrates a complex defence strategy that bolsters microbial resistance and promotes ecosystem restoration.

***ROOT EXUDATES AND MICROBIAL INTERACTIONS***

Root exudates are essential facilitators in the interaction between plants and rhizobacteria. The exudates comprise amino acids, organic acids, sugars, and secondary metabolites that attract beneficial microorganisms and facilitate their colonisation [2]. The rhizobacteria metabolise these molecules, releasing substances that promote growth and provide protection. This dynamic interaction improves pollutant absorption and breakdown, hence facilitating the phytoremediation process. The rhizosphere is frequently characterised as a hub of microbial activity because to the ongoing interchange of signalling molecules and metabolites. Plants provide chemicals that nourish bacteria and act as chemical signals for biofilm formation and quorum sensing. Rhizobacteria, in turn, activate plant defence mechanisms and influence root structure via hormone synthesis and nutrient solubilisation.

Specific exudates also promote the expression of degradation genes in rhizobacteria, thereby synchronising microbial metabolism with environmental requirements. For instance, flavonoids released by legumes can enhance the expression of nitrogen-fixation genes, whereas phenolics and organic acids may activate metal-resistance operons. This bidirectional signalling generates a responsive and robust rhizosphere, adept at responding to varying pollution levels. Moreover, microbial consortia frequently demonstrate synergistic interactions that enhance bioremediation efficacy. Co-cultures of rhizobacteria and mycorrhizal fungi have shown enhanced breakdown rates for complex organic contaminants. These collaborative systems present interesting approaches for integrated phytomicrobial remediation solutions, especially in locations affected by mixed pollutants.

***RHIZOBACTERIAL STRAINS FREQUENTLY UTILISED IN BIOREMEDIATION***

Numerous rhizobacterial species have been thoroughly investigated for their bioremediation capabilities. Illustrations comprise:

- *Bacillus subtilis*: Recognised for its biosurfactant production and endospore formation, which improve resilience in adverse environments. It demonstrates exceptional ability for heavy metal biosorption and hydrocarbon decomposition. Furthermore, it can enhance plant drought resilience, rendering it advantageous in climate-impacted remediation initiatives.
- *Pseudomonas fluorescens*: Proficient in the degradation of organic contaminants such as toluene and naphthalene. It harbours several catabolic plasmids and may express a wide array of oxygenases, facilitating the degradation of chlorinated chemicals, herbicides, and dyes.
- *Azospirillum brasilense*: Engaged in nitrogen fixing and enhancing plant growth under metal stress conditions. It increases root surface area and is associated with enhanced absorption of lead and zinc when co-inoculated with metal-tolerant flora.

- *Rhizobium leguminosarum*: Establishes nodules with legumes and is crucial to nitrogen cycling. Recent investigations demonstrate its capacity to absorb arsenic and augment plant tolerance to metalloids.
- *Enterobacter cloacae*: Renowned for synthesising plant growth hormones and exhibiting significant resistance to cadmium and lead. It is also proficient in nitrate reduction and hydrocarbon degradation. Each of these strains exhibits differing levels of pollutant breakdown, enhancement of plant growth, and adaptability to contaminated conditions. Their application as bioinoculants not only rejuvenates soil fertility but also conforms to sustainable and cost-effective environmental remediation approaches. Progress in microbial genomics and synthetic biology persistently refines these strains for improved efficacy and stability across many ecological environments.

The continuous investigation of indigenous rhizobacterial isolates from polluted environments offers a substantial reservoir of functional characteristics and genetic variation. Indigenous microorganisms, acclimated to local stresses, frequently surpass lab-cultured strains and provide ecological benefits in in situ bioremediation initiatives. Future investigations ought to concentrate on multi-strain consortia, systems biology modelling, and modified rhizobacteria to fully exploit the potential of these microorganisms in environmental biotechnology.

## CRISPR–CAS SYSTEMS IN MICROBIAL ENGINEERING

### *SYNOPSIS OF CRISPR–CAS SYSTEMS*

The CRISPR–Cas (Clustered Regularly Interspaced Short Palindromic Repeats–CRISPR-associated proteins) system is an innovative genome-editing technology derived from the adaptive immune systems of bacteria and archaea. It confers prokaryotic organisms with resistance to foreign genetic material, including phages and plasmids, by specifically targeting and cleaving designated DNA sequences [9]. The CRISPR-Cas9 system, a prevalent version, has facilitated accurate and programmable modification of microbial genomes for diverse applications, including bioremediation.

CRISPR-Cas9 functions via a straightforward method wherein a guide RNA (gRNA) directs the Cas9 nuclease to a complementary DNA region for cleavage. This precise editing facilitates the removal, addition, or modulation of genes linked to pollutant degradation, stress resistance, and metabolic engineering [19]. The simplicity, efficiency, and versatility of CRISPR techniques have rendered them essential in synthetic biology and environmental biotechnology.

### *CRISPR INTERFERENCE (CRISPRI) AND CRISPR ACTIVATION (CRISPRA)*

CRISPRi and CRISPRa are modified iterations of the CRISPR system that facilitate gene control without modifying the DNA sequence. CRISPRi employs a deactivated Cas9 (dCas9) protein to inhibit gene expression by obstructing transcription, while CRISPRa enlists transcriptional activators to augment gene expression [7]. In bioremediation, CRISPRi has been employed to suppress non-essential genes that interfere with or obstruct pollution breakdown pathways. Das et al. [12] illustrated the application of CRISPRi in *Pseudomonas putida* to inhibit pathways not associated with hydrocarbon breakdown, thus enhancing metabolic flux for bioremediation. Likewise, CRISPRa has been utilised to induce the expression of dormant or low-expressed genes that code for catabolic enzymes and stress-response proteins [23]. These methodologies enable researchers to optimise microbial responses to environmental cues, facilitating dynamic regulation of pollutant degradation and microbial viability in adverse settings. CRISPRi/a technologies are particularly advantageous in field environments where environmental factors need swift microbial response.

#### *MODIFYING RHIZOBACTERIA UTILISING CRISPR*

The incorporation of CRISPR systems into rhizobacteria presents novel opportunities for augmenting their bioremediation capabilities. *P. fluorescens* and *B. subtilis*, noted for their environmental robustness and pollutant-degrading capacities, have been effectively modified with CRISPR constructs to enhance their performance [20]. CRISPR facilitates the incorporation of innovative metabolic pathways that degrade enduring organic contaminants, including polychlorinated biphenyls (PCBs), dioxins, and polycyclic aromatic hydrocarbons (PAHs). Moreover, resistance genes for heavy metals including mercury, lead, and arsenic have been incorporated into rhizobacterial genomes, enabling their survival in contaminated soils [16].

A further interesting strategy entails the creation of biosynthetic gene clusters that govern biosurfactant synthesis. These chemicals promote the solubilisation and mobilisation of hydrophobic contaminants, improving their bioavailability and degradation rates. Utilising CRISPR to enhance these clusters in rhizobacteria augments their remediation effectiveness across diverse soil types.

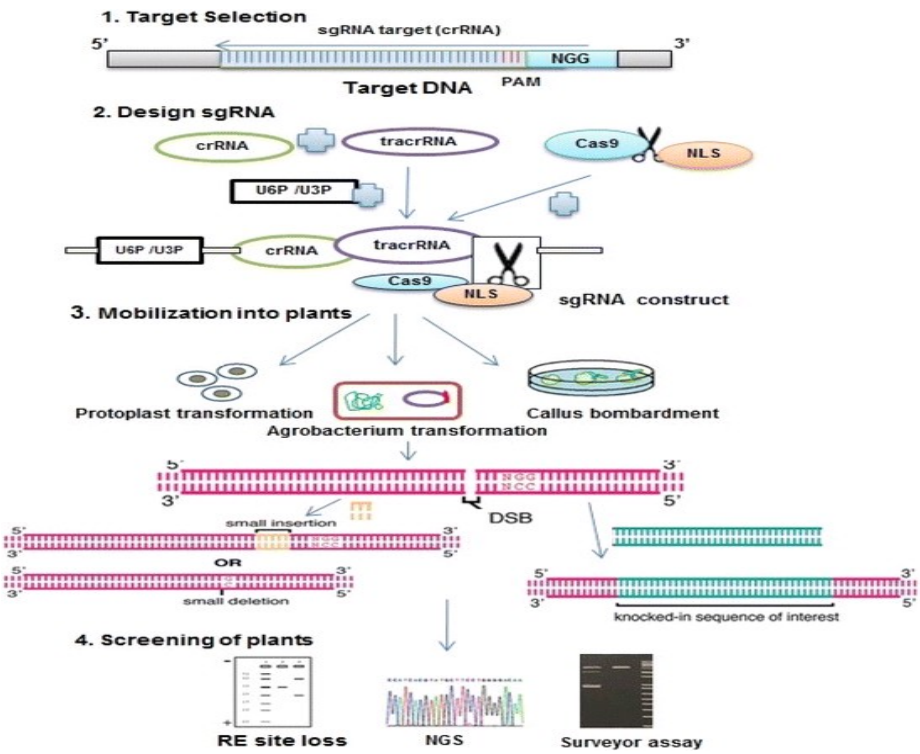


**Figure 2.** Engineering workflow for rhizobacteria [16].

### 2.3 CONCEPTUAL SYNERGY BETWEEN RHIZOBACTERIA AND CRISPR

The amalgamation of CRISPR genome editing with rhizobacteria-driven bioremediation techniques signifies a groundbreaking advancement in environmental biotechnology. Rhizobacteria are inherently proficient in pollutant degradation; nevertheless, their efficacy can be markedly enhanced with the application of targeted genetic alterations utilising CRISPR systems [4]. This synergy facilitates the optimisation of metabolic pathways, stress-response mechanisms, and biosurfactant synthesis, hence augmenting the bacteria's remediation capability [20].

CRISPR-Cas techniques enable accurate modification of genes linked to pollution detection, absorption, and degradation. Researchers have incorporated synthetic operons into rhizobacteria to enhance their capacity to degrade a wider array of organic contaminants, including hydrocarbons, dyes, and pesticides [12]. These improvements enhance the efficiency of rhizobacteria in field conditions, especially in contaminated soils with intricate pollutant profiles. Biosurfactants are surface-active agents synthesised by microorganisms that improve the solubility and mobility of hydrophobic contaminants in soil environments. Scientists have enhanced the biosynthetic pathways for biosurfactant production in rhizobacteria, including *B. subtilis* and *P. fluorescens*, by CRISPR-mediated genetic engineering [23]. These modified strains exhibit elevated emulsification indices, facilitating more efficient degradation and absorption of petroleum hydrocarbons and polycyclic aromatic hydrocarbons (PAHs). CRISPR facilitates the incorporation of new biosurfactant genes from various microbial species, hence enhancing the chemical diversity and applicability of created biosurfactants [20].

Environmental variability presents a considerable obstacle for bioremediation efforts. Researchers are developing CRISPR-based genetic circuits that react to specific environmental stimuli, like pH, temperature, or pollutant concentration [15]. These adaptive systems enable rhizobacteria to initiate pollutant-degrading genes solely in the presence of pollutants, thereby conserving cellular energy and enhancing survival. A recent study developed *Pseudomonas* strains using a CRISPR-responsive circuit that activates hydrocarbon-degrading enzymes upon exposure to benzene and toluene, yielding a 40% enhancement in bioremediation efficacy under simulated soil conditions [16]. The dynamic regulation of gene expression improves the robustness and reliability of rhizobacterial remediation techniques in fluctuating environmental conditions. Although laboratory-scale investigations have shown considerable effectiveness with CRISPR-engineered rhizobacteria, outdoor trials introduce further complications. Soil heterogeneity, microbial competition, and variable environmental conditions can restrict the effectiveness of modified strains [14]. Comparative evaluations indicate that strains optimised in controlled settings frequently necessitate further modifications or support systems to achieve consistent performance in natural contexts. Pilot studies in contaminated agricultural lands in Asia and Africa demonstrate that CRISPR-enhanced strains of *B. subtilis* and *P. fluorescens* significantly surpass their wild-type equivalents in diminishing heavy metal bioavailability and degrading pesticide residues [21]. These findings highlight the potential of combining CRISPR systems with native microbial communities for localised bioremediation efforts.

## METHODOLOGICAL APPROACHES FOR CRISPR ENGINEERING OF RHIZOBACTERIA

### SELECTION OF TARGET STRAINS AND GENES

The initial phase of CRISPR-mediated engineering of rhizobacteria involves the intentional selection of strains demonstrating ecological compatibility and pollutant degrading proficiency [3, 16] (Figure 3).

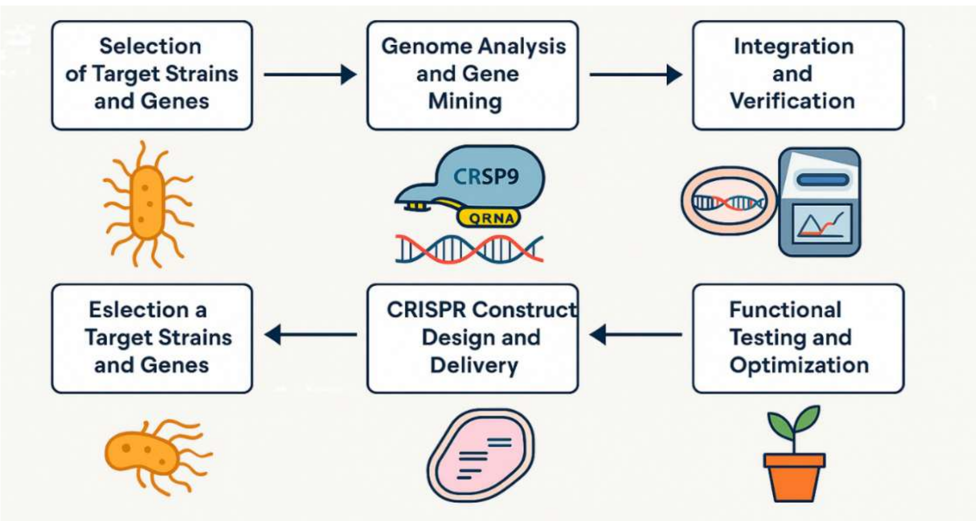


**Figure 3.** Methodological approaches for CRISPR engineering of Rhizobacteria [16].

Prominent strains such *P. fluorescens*, *B. subtilis*, and *A. brasilense* are well-documented for their symbiotic interactions with plant roots and their vigorous metabolic functions under environmental stress [3, 16]. These rhizobacteria inherently possess the ability to detoxify soil pollutants via mechanisms including biosurfactant synthesis, enzymatic degradation, and biofilm development [3].

Scientists generally prioritise target genes associated with hydrocarbon breakdown, heavy metal reduction, and oxidative stress tolerance. Genes that code for enzymes like monooxygenases, dioxygenases, laccases, and reductases are prominent candidates [23]. Regulatory genes linked to quorum sensing, nutrition acquisition, and stress-response mechanisms are also targeted for alteration or overexpression with CRISPR. Bioinformatics tools such as KEGG, RAST, and NCBI Genome Browser enhance the functional annotation of genomic data, allowing researchers to identify high-value targets [23].

A crucial factor in strain and gene selection is the possibility for horizontal gene transfer. Rhizobacteria in soil ecosystems frequently interact with various microbial populations, and their capacity to preserve stability while preventing inadvertent gene transfer is essential [19]. Consequently, strains exhibiting established genetic stability and minimal natural transformation rates are favoured [12]. Host compatibility constitutes another essential aspect. Effective CRISPR-mediated gene editing relies on the congruence between the bacterial genome and the modified CRISPR-Cas components [7]. Promoter strength, codon use, and restriction-modification mechanisms affect the efficacy of gene insertion and expression [19]. Researchers frequently utilise pre-validated CRISPR toolkits tailored for Gram-negative or Gram-positive bacteria to improve editing efficacy [20]. Additionally, selection markers (such as antibiotic resistance or fluorescent genes) and regulatory elements (including robust endogenous promoters) are incorporated into CRISPR constructs during the design phase to facilitate the identification of successful transformants. These factors jointly influence the accuracy, stability, and safety of the genome editing process in environmental situations [23].

## GENOMIC ANALYSIS AND GENE EXPLORATION

Genome sequencing of rhizobacterial candidates establishes a foundation for precise gene editing. High-throughput technologies, like Illumina and Oxford Nanopore, provide whole-genome sequencing, offering an extensive overview of coding and non-coding areas, mobile genetic elements, and metabolic pathways [19]. Annotated genomes indicate the existence of biosynthetic gene clusters (BGCs) associated with pollution degradation and metal resistance [12].

Comparative genomics facilitates the identification of conserved and strain-specific genes across rhizobacteria sourced from diverse environments [12]. This analysis identifies homologous genes associated with bioremediation and provides insight into gene loss or acquisition that may affect functional efficacy [7]. Pan-genome analyses enhance this methodology by elucidating the core, auxiliary, and distinct genes among bacteria acclimated to polluted environments. Functional gene mining encompasses not only genomics but also transcriptomics and proteomics [23]. Researchers can discover differentially expressed genes and proteins under pollutant exposure using RNA-seq and LC-MS. This encompasses genes that code for metallothioneins, siderophores, and detoxification enzymes, including superoxide dismutase and glutathione S-transferase [19]. In silico analyses are frequently corroborated by experimental validation. Gene knockdown or overexpression experiments confirm the functions of target genes in pollution detoxification [20]. Phenotypic experiments performed in pollutant-contaminated growth medium facilitate the correlation of gene function with bioremediation efficacy [19].

In addition to individual genes, researchers identified synthetic pathways and regulatory circuits that may be reengineered [12]. Metabolic flux analysis (MFA) and genome-scale metabolic models (GEMs) are utilised in systems biology to predict the impact of gene expression alterations on metabolic output [20]. These models are essential for forecasting the impacts of CRISPR changes before to experimental execution [23]. Furthermore, metagenomic sequencing of contaminated rhizospheres reveals previously uncharacterized microbial taxa and genes with potential bioremediation applications [9] Environmental records are analysed for CRISPR spacers, resistance genes, and catabolic pathways that could be included into genetically manipulable rhizobacterial hosts [23].

**DESIGN AND DELIVERY OF CRISPR CONSTRUCTS**

Upon identification of gene targets, the construction of CRISPR constructs necessitates the meticulous assembly of guide RNAs (gRNAs), Cas effectors, promoters, and selection markers [20]. The gRNAs are engineered to identify specific DNA sequences near protospacer adjacent motifs (PAMs), guaranteeing accurate targeting [19]. Instruments such as CRISPRdirect, CHOPCHOP, and Benchling enable researchers to identify gRNAs with reduced off-target risks and enhanced cleavage efficiency [23].

The CRISPR system can be tailored for gene knockout (CRISPR-Cas9), base editing (Cas9 nickases), or transcriptional regulation (CRISPRi/CRISPRa), depending on the intended result [7]. Researchers are increasingly favouring CRISPRi and CRISPRa for environmental applications because of their reversibility and less ecological danger [20]. These approaches employ catalytically inactive Cas variants to modulate gene expression without modifying genomic DNA [23]. Vectors employed for CRISPR delivery must be compatible with the target strain [12]. Plasmid vectors are typically employed for transitory expression, whereas integrative vectors or phage-derived systems provide stable chromosomal insertion [19]. Broad-host-range plasmids such as RK2 and pBBR1MCS are favoured for Gram-negative rhizobacteria [23]. For Gram-positive bacteria, shuttle vectors possessing an origin of replication functioning in both *Escherichia coli* and rhizobacteria are utilized [20].

CRISPR constructs are delivered into rhizobacteria using techniques such as electroporation, heat-shock transformation, and conjugation [20]. Electroporation is preferred due to its great efficiency and repeatability, however it necessitates meticulous regulation of voltage and ionic strength [20]. Conjugation facilitates the transfer of plasmids among several bacterial species, rendering it appropriate for field isolates exhibiting poor natural competence [19]. To improve transformation efficiency and stability, supplementary components including ribosome binding sites (RBS), terminators, and CRISPR arrays are tailored for each host [20]. Additionally, safety switches like as inducible promoters and toxin-antitoxin systems are incorporated to mitigate the possibility of unregulated expression or environmental dissemination [23]. Post-transformation, altered bacteria are evaluated using selective media and confirmed via colony PCR, sequencing, and fluorescence imaging [12]. Functional assays are performed to verify that the designed characteristics (e.g., pollutant degradation, heavy metal chelation) are preserved and enhanced [23]. These assessments replicate authentic conditions, encompassing fluctuations in pH, temperature, and soil moisture [20]. The creation and delivery of CRISPR constructs constitute a vital stage in the bioengineering process [23]. An effective CRISPR construct harmonises specificity, efficiency, and ecological safety, guaranteeing that the modified rhizobacteria function reliably in environmental settings for sustainable bioremediation purposes [20].

**INTEGRATION AND VERIFICATION**

After the successful introduction of CRISPR constructs into rhizobacteria, it is essential to verify the incorporation of genetic elements to confirm targeted genome editing [23]. Polymerase Chain Reaction (PCR) and Sanger sequencing are conventional methodologies employed to confirm the existence and precision of inserted or changed genes [20]. These techniques enable researchers to identify insertion locations, ascertain the lack of off-target effects, and validate mutation types [19]. Southern blotting can be employed in cases of chromosomal integration, especially to differentiate between single and numerous insertions [12]. Furthermore, reverse-transcription PCR (RT-PCR) is utilised to measure the expression levels of newly integrated or regulated genes [23]. Fluorescent reporter genes, including GFP and RFP, are frequently utilised in conjunction to visually verify effective gene expression in modified cells [20]. Additionally, CRISPR-modified rhizobacteria undergo plasmid stability evaluations and growth curve assessments under both optimal and stress settings to determine metabolic expenditure and ecological viability [7]. These verification procedures confirm that the targeted features are genetically stable and operational under conditions pertinent to bioremediation [16].

### FUNCTIONAL TESTING AND OPTIMISATION

Functional testing is the stage in which modified rhizobacteria are assessed for improved pollutant degradation and environmental adaptability [3]. This involves utilising contaminant-spiked media to replicate field circumstances, whereby growth rate, enzymatic activity, and degradation efficacy are assessed [17]. Substances like hydrocarbons, herbicides, or heavy metals are put into growing settings to evaluate real-time detoxification [9].

Enzyme assays for monooxygenases, laccases, and reductases are employed to measure degradation kinetics [20]. Inductively coupled plasma mass spectrometry (ICP-MS) and high-performance liquid chromatography (HPLC) are utilized to identify residual contaminants or their by-products [23]. Microcosm and mesocosm studies that replicate field conditions are established to examine microbial behaviour in authentic soil or hydroponic systems [12]. These trials assess variables such as soil pH, plant growth metrics, microbial community alterations, and nutrient cycling [3]. Optimisation occurs after to functional validation and include adjusting promoter strength, gRNA sequence specificity, or the copy number of constructs to enhance performance [20]. Bioinformatics methods such as metabolic flux analysis (MFA) and flux balance analysis (FBA) aid in forecasting and optimising gene expression to enhance bioremediation yield [23]. This technique ultimately converts promising modified strains into feasible candidates for field application.

## CONSIDERATIONS FOR ENVIRONMENTAL AND BIOSAFETY

### OFF-TARGET EFFECTS

CRISPR-Cas systems, while extremely specific, may demonstrate off-target activity resulting in the cleavage or modification of undesired genomic regions. This inadvertent alteration may disrupt critical genes, thereby jeopardising microbial viability or modifying ecological relationships [19]. In rhizobacteria, off-target impacts may diminish pollutant degradation efficacy or disrupt plant-microbe symbiosis [23]. Methods including whole-genome sequencing (WGS), GUIDE-seq, and deep sequencing technologies are frequently utilised to identify off-target alterations across the genome [20]. These tools assist scientists in assessing unwanted modifications and optimising CRISPR designs accordingly. The advancement of high-fidelity Cas9 variants, including SpCas9-HF1 and eSpCas9, has markedly diminished these instances by improving DNA targeting specificity [19]. However, the potential for unforeseen modifications poses an obstacle to practical implementations, necessitating ongoing evaluation and refinement to guarantee that modified strains fulfil their designated bioremediation roles without adverse consequences. Moreover, computational prediction methods like CRISPRoff and CRISPRitz are utilised to pre-assess off-target hazards prior to experimental implementation [23]. Integrating computational predictions with experimental validations provides a solid framework to mitigate unwanted outcomes.

## BIOCONTAINMENT STRATEGIES

Biocontainment strategies seek to avert the escape, persistence, and inadvertent ecological consequences of genetically modified rhizobacteria in non-target ecosystems [23]. Due of the impracticality of physical containment in open-field applications, researchers have created genetic and metabolic safeguards to mitigate dangers [20]. One of the most promising strategies is genetic kill-switches, which entail the incorporation of deadly genes that are triggered by certain environmental signals or the lack of externally provided inducers [19]. This guarantees that the altered organism terminates itself beyond specified areas. An alternative method involves the development of auxotrophic mutants that necessitate uncommon nutrients absent in natural soils, thereby restricting their viability in external habitats [23].

Moreover, researchers employ toxin-antitoxin systems and quorum-sensing feedback mechanisms to regulate population development and dissemination in uncontrolled environments [23]. These methodologies enhance security and are frequently employed in conjunction to guarantee resilience. The efficacy of biocontainment is generally assessed in microcosms and mesocosms-controlled environments that replicate field conditions-prior to full-scale implementation [12]. Innovative methods encompass synthetic gene circuits featuring multi-tiered logic gates capable of detecting environmental stimuli and subsequently initiating degradation pathways or confinement responses [20]. Nonetheless, despite these developments, no technique ensures complete containment. Consequently, regulatory standards advocate for the integration of several fail-safe systems.

## RISK OF LATERAL GENE TRANSFER

Horizontal gene transfer (HGT) denotes the transmission of genetic material among unrelated organisms and poses a considerable biosafety risk for modified rhizobacteria utilised in environmental applications [19]. This mechanism may disseminate tailored characteristics, including antibiotic resistance or CRISPR designs, to non-target microbial communities [23]. The rhizosphere is especially susceptible owing to its elevated microbial density and dynamic gene exchange milieu [3]. Plasmids, transposons, and integrative conjugative elements are prevalent vectors that enable gene transfer within these microbial populations [23]. Such exchanges can modify native microbial functions, diminish biodiversity, or induce ecological imbalances. To alleviate these hazards, engineered constructs are frequently built with non-mobile plasmid backbones, and genetic integration is precisely targeted to chromosomal sites to reduce mobility. Additionally, researchers are investigating site-specific recombinases and orthogonal gene expression systems to restrict horizontal compatibility [20].

Real-time monitoring of horizontal gene transfer (HGT) employs techniques such as quantitative PCR (qPCR), digital PCR, and next-generation metagenomic sequencing [7]. These methodologies provide accurate monitoring of modified genes inside intricate settings. Risk evaluations now incorporate modelling of gene flow and transfer probabilities across several environmental matrices.

In summary, although techniques to mitigate horizontal gene transfer (HGT) are available, their efficacy depends on rigorous genetic design and post-deployment surveillance to guarantee ecological stability and safety.

## REGULATORY AND ETHICAL CONSIDERATIONS

The deployment of CRISPR-engineered rhizobacteria for environmental bioremediation presents many regulatory and ethical challenges. Regulatory frameworks vary markedly between nations, encompassing both stringent prohibitions and conditional permissions contingent upon biosafety assessments [16]. In numerous jurisdictions, regulatory monitoring encompasses biosafety agencies, including the Environmental Protection

Agency (EPA), genetic modification review boards, and institutional biosafety committees [20]. These entities evaluate the possible environmental, health, and ecological hazards of modified organisms prior to sanctioning field trials or commercialisation.

Ethical issues centre on environmental justice, possible unforeseen ecological repercussions, and social approval for the introduction of GMOs in communal areas. The public's perception and approval are crucial for the adoption of these technologies. Thus, global ethical norms advocate for stakeholder participation, transparent communication, and participatory decision-making [23].

Moreover, there are philosophical discussions over human meddling in natural microbial communities. Some advocate for bioengineering as a solution to pollution concerns, while others warn about potential unexpected long-term implications [9]. These talks underscore the necessity for a balanced methodology that amalgamates precautionary ideals with scientific advancement. There is a growing advocacy for international cooperation and unified regulatory norms to prevent fragmented oversight and encourage responsible innovation [23]. Organisations like the OECD and WHO have commenced discussions on global governance regarding genome-organisms in environmental biotechnology. Ultimately, the ethical implementation of CRISPR-modified rhizobacteria necessitates interdisciplinary collaboration among scientists, ethicists, policymakers, and the public to establish frameworks that are scientifically robust, socially acceptable, and ecologically sustainable [12].

## CONCLUSIONS

CRISPR-based engineering has emerged as a transformative instrument in environmental biotechnology. By enabling scientists to precisely edit and manipulate rhizobacterial genomes, CRISPR amplifies the potential for bioremediation to transition from theoretical concepts to practical field applications. Engineered rhizobacteria provide a comprehensive approach to pollutant management, particularly in compromised soils and ecosystems, by enhancing pollutant degradation, stress resilience, and synergistic interactions with plants. Nonetheless, these encouraging results are accompanied with biosafety and ethical concerns that must be addressed. Off-target modifications, the risk of unregulated gene dissemination, and the lack of cohesive worldwide regulatory frameworks persist as significant obstacles. Moreover, social attitudes and the imperative of ethical governance underscore the significance of transparency and interdisciplinary collaboration in research and implementation.

**Author Contributions**: Conception of study SCO; Drafting manuscript SCO; Critical revision of manuscript DMD; Final approval and accountability SCO and DMD; Supervision SCO. All authors have read and agreed to the published version of the manuscript.

**Conflict of Interest:** The authors declare no potential conflict of interest.

**Source of Funding:** This work has not received any financial support.

**Ethical Statement:** This study does not contain any studies with human subjects or animals.

**Data availability:** All the data supporting the results of this study are included in the article.

## REFERENCES

1. Ali SS, Al Tohamy R, Li F, Okasha KM, Mahmoud YAG, Mahmoud RH, Helmy OM, Zhang R. Environmental remediation through microbial interventions: recent advances and future challenges. Front Microbiol. 2021;12:655172.
2. Sharma B, Dangi AK, Shukla P, Varjani S. Bioremediation: a sustainable approach for pollution control. Environ Monit Assess. 2020;192(9):1-22.

3. Hashem A, Tabassum B, Fathi Abd Allah E, Al-Arjani ABF, Egamberdieva D. Rhizobacteria and their role in bioremediation of heavy metals. Ecotoxicol Environ Saf. 2021;213:112067.
4. Bhatt P, Singh R, Sharma P. CRISPR-Cas: a potential tool for sustainable bioremediation. J Environ Manage. 2021;284:112006.
5. Bharagava RN, Sharma B, Kumar A, Mishra S. Role of rhizobacteria in bioremediation of soil pollutants. Environ Chem Lett. 2020;18:889-907.
6. Kumar M, Sharma P, Singh R. Engineering plant-microbe interactions for enhanced bioremediation using CRISPR/Cas9. J Appl Microbiol. 2020;129(4):827-838.
7. Jiang W, Bikard D, Cox D, Zhang F, Marraffini LA. Applications of CRISPR technology in bioremediation. Trends Biotechnol. 2021;39(7):673-676.
8. Zhang C, Zhang H, Wang L, Xu T, Zhang J. The use of nanotechnology in enhancing plant productivity and its compatibility with arbuscular mycorrhizal fungi. J Nanobiotechnology. 2020;18(1):1-15.
9. Rashid MI, Mujtaba M, Mehmood M, Waqas M. CRISPR-Cas systems: versatile genome editing tools for bacteria in environmental biotechnology. Biotechnol Adv. 2021;49:107763.
10. Chaudhary DK, Varma A, Mishra S. CRISPR-Cas systems in environmental biotechnology: emerging applications and challenges. Environ Pollut. 2021;276:116724.
11. Sahu R, Das S, Patel N. CRISPR tools for microbial degradation of organic pollutants. Sci Total Environ. 2022;815:152798.
12. Das AP, Pandey R, Bhardwaj A, Ghosh A. Microbial engineering through CRISPR-Cas: new horizons in environmental biotechnology. Biocatal Agric Biotechnol. 2023;47:102559.
13. Patel P, Sharma R, Verma S. Potential of rhizospheric microbes in phytoremediation: genetic enhancement through CRISPR. Environ Nanotechnol Monit Manag. 2021;16:100506.
14. Ma J, Zhang W, Li Y, He J. Barriers to genetic engineering in soil microbiomes. Appl Microbiol Biotechnol. 2020;104:4027-4037.
15. Nayak AK, Panda S, Behera BC, Das D. Responsive genetic circuits in rhizobacteria for environmental bioremediation. Synth Syst Biotechnol. 2022;7(2):532-542.
16. Rana A, Yadav S, Verma A, Singh J. Enhancing phytoremediation via CRISPR-engineered rhizobacteria: a new paradigm. J Plant Interact. 2023;18(1):50-63.
17. Srivastava P, Choudhary DK, Sharma S, Singh J, Singh V. Microbial biosurfactants in remediation of heavy metals and hydrocarbons. Environ Chem Lett. 2021;19(3):2133-2150.
18. Sarkar A, Ghosh S, Chatterjee A, Chattopadhyay D. Biosafety of CRISPR-Cas edited microbes for environmental applications. Crit Rev Biotechnol. 2021;41(2):213-230.
19. Lee J, Park S, Kim H. CRISPRi and CRISPRa: emerging tools for microbial metabolic engineering and synthetic biology. ACS Synth Biol. 2021;10(1):5-15.
20. Gupta S, Tiwari S, Verma H, Prasad R. CRISPR-based gene regulation for bioremediation: a review on present and future perspectives. Mol Biotechnol. 2022;64(9):891-907.
21. Nwachukwu OI, Eze CI, Okeke CC. Environmental biotechnology in Africa: exploring the CRISPR frontier. Environ Manage. 2021;67:350-359.
22. Kumar S, Patel R, Mehta A. CRISPR and microbial remediation: genomic tools for future environmental clean ups. Environ Res. 2021;201:111565.
23. Fatima K, Shakoor S, Ahmed S, Ameen A. Harnessing CRISPR-based genome editing in bacteria for bioremediation: promises and perils. Environ Sci Pollut Res Int. 2023;30:45678-45695.