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Bioremediation Potential of CRISPR Technology

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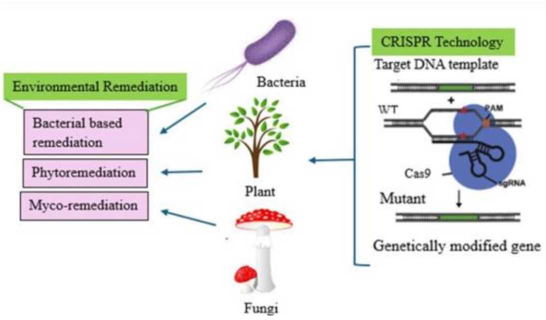
Graphical abstract



Abstract

Environmental pollution, linked to pollutants such as heavy metals (HMs), disposal generated from plastic materials, pesticides, and other degradable and nondegradable wastes, has serious consequences for ecosystems and human well-being. Environmental pollutants mostly caused by HMs are the major driving force for different levels of illnesses in humans, including neurological defects, respiratory and cardiovascular illnesses, as well as cancer. The cost of health care expenses and costs related to the remediation activity are a significant economic downturn arising from environmental pollutants. Environmental pollutants are also responsible for the decrease in agricultural output. Traditional bioremediation measures, which utilize plants (phytoremediation) and microbes (microbial bioremediation), are often limited by slow degradation rates and lack the ability to target specific pollutants. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) is one of the emerging bioremediation approaches globally. This approach has several advantages over conventional approaches. Despite its relevance, CRISPR-mediated bioremediation faces ecological constraints, ethical and regulatory concerns, and potential off-target effects. This review demonstrates the potential of CRISPR technology in the bioremediation of environmental pollution, highlights the potential of CRISPR-based bioremediation, and provides prospects in environmental rehabilitation.

Keywords: CRISPR Technology, Environmental pollution, Bioremediation, Xenobiotics

1. Introduction

Environmental pollution by the so-called ‘new generation’ or ‘persistent’ pollutants and xenobiotics has been regarded as a leading concern for the global environment and human wellbeing. Several types of contaminants, including polychlorinated compounds, heavy metals (HMs), plastics, and various agro-industrial chemical wastes, are the key pollutants of the environment due to their non-biodegradable and toxic nature. Bioremediation is one of the most reliable, eco-friendly, and cost-efficient approaches for cleansing toxicants in polluted environments and is emerging as the most viable approach to restoring degraded environments and safeguarding the ecosystem at large (Bala *et al.* 2022). This process exploits biological agents, chiefly plants, microorganisms—e.g., fungi, bacteria—to detoxify and

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remove pollutants from soil, water, and air. Unlike traditional approaches, microbial-based bioremediation has been shown to convert toxic contaminants to less harmful products via natural metabolism, making them safer and more convenient for the existing ecosystem. Bioremediation can address a diverse range of pollutants, including organic pollutants, hydrocarbons, and HMs, thereby playing a key role in mitigating environmental deterioration (Kumara *et al.* 2023).

Traditional bioremediation, such as bio-stimulation and bioaugmentation, has been chiefly employed to sustain the biodegradation activity of indigenous microbial populations (Liu *et al.* 2023). Bioaugmentation involves introducing strains of microbial agents that are vital for degrading pollutants prolifically, whereas bio-stimulation facilitates adding nutrients or adjusting ecological dynamics to trigger the bioprocess of microbes (Chettri *et al.* 2024; Muter 2023). Parallel to this situation, phytoremediation harnesses plants to detoxify, aggregate, and absorb pollutants from water, and soil can be employed to alleviate environmental degradation. Despite the fruitfulness of these traditional strategies, they commonly confront obstacles such as restricted effectiveness towards complex hazardous agents, slow breakdown rates, and sensitivity to environmental factors—such as temperature, pH, and the presence of toxic compounds—that can restrict the biodegradation potential of microbial agents (Bartucca *et al.* 2023).

The emergence of genetic engineering (GE) has considerably increased the potential of bioremediation. Through the implementation of genetic engineering, expertise can transform strains with the capacity to disintegrate specific pollutants to endure unfavorable environmental conditions (Rafeeq *et al.* 2023). To this end, GE can transform organisms to metabolize complex and recalcitrant pollutants resourcefully, thus introducing cutting-edge remediation systems. It also assists in developing tailored solutions targeting special contaminants and hence promotes the positive performance of bioremediation. The collaboration of GE with bioremediation hastens a diverse range of limitations of traditional platforms and brings new tactics for managing serious environmental pollutants (Arunraja *et al.* 2023).

CRISPR technology is an emerging GE tool that has been employed to generate precise genetic re-arrangements in microorganisms to maximize their performance for radical protection of the environment (Sahoo *et al.* 2022). Using CRISPR/Cas systems, one can engineer microbes of the natural ecosystem to degrade a pollutant or counteract the toxic constituent of the pollutant. For instance, CRISPR has been used to create improved bacterial strains with enhanced capabilities to detoxify HMs and petroleum hydrocarbons into their useful elements in contaminated environments, thus potentiating the bioremediation technologies for contaminated sites (Wijegunawardana *et al.* 2022; Perera *et al.* 2023). In light of published findings, integrated approaches (e.g., biosensors) can upgrade the detection power of CRISPR technology, thereby enabling timely, efficient, and viable monitoring of environmental pollution (Chen *et al.* 2022).

The aim of this review is to provide an overview of the role of CRISPR/Cas9 technology in microbial-based biodegradation of pollutants applied for the biodegradation of toxic environmental pollutants released to the ecosystem. Besides, the present limitations and future direction will be explored.

2. Environmental Pollution

Pollution of the environment—i.e., soil and fresh water—has risen with the industrialization of the world. Some human activities, such as mining and the emission of some hazardous metal effluents from steel mills, power plants, and related factories, have presumably altered the water quality and thus brought out some serious ecological constraints (Shah 2021).

Urbanization and industrialization are the key driving forces for environmental pollution, which is mainly associated with the intentional or unintentional leakage of toxic chemicals into the ecosystem. This situation is mainly caused by different sectors, including the manufacturing sector—e.g., detergent and dye production—, mining activities, and the construction sector—e.g., metal and cement industries. The consumption of pesticides, herbicides, and fertilizers in agriculture brings HMs pollution linked to nickel, copper, lead, zinc, arsenic, and aluminum into the environment (Ayilara *et al.* 2021). Untreated contaminants from the wastewater of agriculture and food manufacturing companies, when released into water bodies, exerted detrimental consequences on the biotic and abiotic components of the existing environment (Tariq and Mushtaq 2023). Industrial or non-industrial waste that comes out in effluents includes plastics, petroleum, and trace metals, which are considered potential environmental poisons. If these pollutants are emitted into the ecosystem at high levels, they can remain toxic for a rather long time, thus complicating the state of pollution and bioremediation processes (Akpor *et al.* 2014). Many of these compounds are mutagenic and represent potential health threats to both humans and the environment.

Heavy metals, once assimilated, tend to precipitate in the kidneys, liver, and brain. In animals, these metals can result in cancer, restricted growth, and deterioration to the nervous system, and finally lead to death (Briffa *et al.* 2020). Air contaminating agents—e.g., nitrogen oxides, which are released in response to combustion activities, can be inhaled by individuals and create respiratory illnesses accompanied by coughing, short breathing, and asthma (Lee *et al.* 2021). Likewise, fossil fuel byproducts—e.g., sulfur dioxide—are the driving force for respiratory and cardiovascular illnesses in humans, as seen in serious fatal respiratory cases reported in China (Li *et al.* 2021). Toxic organic volatile chemical constituents, which are mainly emitted from vehicles, paints, and cleaning agents, can also expose humans to respiratory illnesses. One such example of these products is benzene, which is responsible for lung cancer in humans (Ratiu *et al.* 2020).

Likewise, according to documented findings, most animal illnesses are initiated by pollutants (Chaitanya *et al.* 2024). Consuming water polluted with pesticides, pharmaceuticals, and HMs can result in deterioration of multiple organs and systems, including liver damage, reproductive disorders, and cancer in animals (Singh *et al.* 2022). Pollutants in the environment restrict plant growth and shrink crop yields, whereas sulfur dioxide is liable for acid rain and acidification (Luo *et al.* 2019). Plants exposed to ozone face alterations in metabolism and biochemical activities (Tiwari *et al.* 2018). In aquatic creatures, high nitrogen amounts result in eutrophication, leading to algal blooms that impact the health of fish, degrading their diversity and causing death (Shahmohamadloo *et al.* 2023).

The global fiscal effect of environmental pollution is serious, entailing a significant financial burden caused by the destruction of ecosystems, public health, and infrastructure. Pollution is significantly linked to various health problems, mainly but not limited to cardiovascular and respiratory tract diseases, which can lead to cancer or other fatal cases. A polluted environment also degrades the viable components of the ecosystem and can lead to a reduction in agricultural products (fish, vegetables, crops, production), which in turn contributes to food insecurity and economic disability.

2.1. Heavy metal pollution

Heavy metals are metallic elements that have a significantly higher density than water (Yu *et al.* 2023). Around 25% of Earth's mass is composed of metals. Above fifty elements on the periodic table have been recognized as HMs, which comprise metalloids, transition metals, lanthanides, and actinides. Some of these elements include mercury (Hg), strontium (Sr), zinc (Zn), lead (Pb), boron (B), copper (Cu), nickel (Ni), cobalt (Co), vanadium (V), titanium (Ti), molybdenum (Mo), arsenic (As), cadmium (Cd), and chromium (Cr). Nearly 17 of these HMs are categorized as acutely life-threatening and relatively accessible. It is obvious that selected HMs such as boron, nickel, iron, copper, and zinc are crucial for the growth of plants in small amounts, yet, at levels exceeding permissible concentration, they become toxic to humans, animals, and plants (Okpara *et al.* 2022). Because of their abundance, metals have a wide range of applications (Aureliano *et al.* 2023). The recurrent utilization and repeated exposure of humans to HMs have increased the risk of internalization into the body of humans. The toxicity of a HMs is often associated with its mass and harmful characteristics, which are typically interconnected (Tchounwou *et al.* 2012). The European Environment Agency (EEA) has set limit values for soil heavy metal levels, including Hg (0.20 ppm), Cd (0.44 ppm), As (0.11 ppm), Pb (0.48 ppm), and Cr (0.20 ppm) (Baritz *et al.* 2023). The World Health Organization (World Health Organization 2002) reported that the permissible limits of HM pollutants in drinking water are as follows: Hg (0.001 ppm), Cr (0.05 ppm), Pb (0.05 ppm), Cd (0.005 ppm), and As (0.05 ppm) (World Health Organization 2002). The WHO, United Nations (UN), and Food and Agriculture Organization (FAO) set the tolerable levels for HM intake from vegetables as follows: Cd (0.2 mg/kg) for leafy vegetables, 0.3 mg/kg for root vegetables, Cr (0.1 mg/kg), Pb (0.15 mg/kg), Hg (0.05 mg/kg), As (0.1 mg/kg) for all vegetables (Sharma *et al.* 2016; Wu 2014; Åkesson *et al.* 2015). As per the heavy metal guidelines set by India, the tolerable levels of HMs concentration in soil are 250–500 mg/kg for Pb, 135–270 mg/kg for Cu, 3–6 mg/kg for Cd, 75–150 mg/kg for Ni, and 300–600 mg/kg for Zn (Ayangbenro *et al.* 2019).

Heavy metal pollution is a serious concern to the environment due to the poisonous nature of the agent to the health of humans and ecosystems. In general, two kinds of metals are predominantly encountered in the polluted sites. The first kind of metals are Cationic metals (positive charge), which are metals such as lead, zinc, mercury, chromium, cadmium, copper, and nickel. In contrast, negatively charged anionic metals are known by their negative charge, where arsenic is the most frequently found anionic element in these environments (Olaniran *et al.* 2013). HMs can enter the ecosystem via various routes, mainly improper waste disposal systems, impurities released from factories, agricultural practices, and mining procedures (Chen *et al.* 2022). Once introduced into the environment, HMs can stay for a sustained period due to their low movement and tendency to gather in soil, water, and sediments. The accumulation of HMs in the food chain causes serious problems, including developmental and neurological sicknesses in humans, and reproductive complications in wildlife (Nkwunonwo *et al.* 2020). The non-degradable behavior of metals and their multifaceted usage have caused bioaccumulation in human body parts via the food chain (Maurya & Malik 2019).

Recent research has demonstrated the widespread pollution of soil and water resources with HMs. For instance, industrial waste and agricultural runoff have been considered as chief sources of HM pollution in diverse areas (Mokarram *et al.* 2020). In urban zones, emissions of traffic and waste from factories play an irreplaceable role in elevated levels of HMs such as Cd and Pb in soils and water bodies (Nazir *et al.* 2015). Controlling and assessing HMs pollution is a key factor for understanding the degree of pollution and for constructing effective remediation techniques to mitigate its adverse effects.

The existence of HMs in soil lessens both the quantity and quality of food by inhibiting plant growth, interfering with nutrient absorption, and metabolic activities. Likewise, the deterioration of natural resources necessitates extensive cleanup and remediation attempts, straining both private and public sector budgets (Zaharia *et al.* 2014).

Metals are essential for health in small quantities, but in large amounts, they can be toxic to human beings. HM toxicity can garble the physiological framework of the organs, thereby triggering chronic and/or acute illness (Sharma *et al.* 2023).

Continuous exposure of individuals to HMs can result in progressive muscular, physical, and neurological illness that can lead to different disorders (Figure 1) and conditions such as Alzheimer's, muscular dystrophy, multiple sclerosis, and Parkinson's disease (Vellingiri *et al.* 2022; Islam *et al.* 2022). Besides, cancer is one of the other conditions that are linked to the prolonged exposure of individuals to HMs (Matés *et al.* 2019).

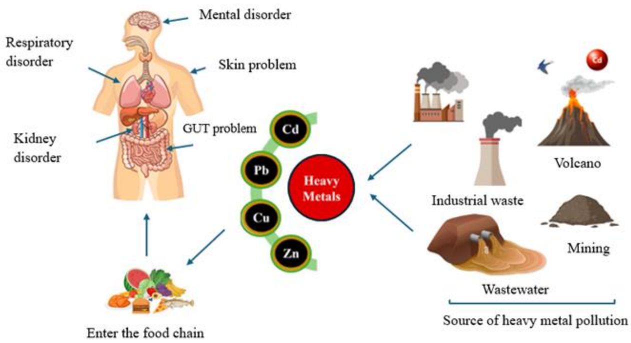

Figure 1. Major source of HMs and the mechanism through which they reach humans, and their health effects.

2.2. Organic pollutants

Several organic pollutants (Ops), including polycyclic aromatic hydrocarbons (PAHs), are the most encountered contaminant agents due to their toxicity, accumulation, and sustained environmental consequences (aquatic and terrestrial ecosystems) (Ruan *et al.* 2023). PAHs, a collection of organic compounds composed of multiple aromatic rings, are largely synthesized by the partial burning of organic matter. They are sourced from simple vehicular emissions to fossil fuel combustion, and compounds released from industrial activities (Kim *et al.* 2020). Organic pollutants are known for their mutagenic and carcinogenic characteristics (Zahed *et al.* 2023).

Organic pesticides are commonly utilized in agroecosystems to control pests and boost crop productivity. However, their wrong utilization of these compounds has led to pollution of multiple units of the ecosystem and human beings. Organochlorine pesticides, such as lindane and DDT, are notorious for their persistence and capability to remain in the food chain, leading to harsh effects on human health, domestic animals, and wildlife (Singh *et al.* 2018). Even recent pesticides, manufactured to degrade quickly, can cause risks if not managed in an organized manner. For instance, neonicotinoids have been linked to a decrease in pollinator populations, raising concerns about their ecological effect (Carrasco-Navarro & Skaldina 2018).

The remediation of environments polluted with OPs necessitates a viable and coordinated struggle of chemical, physical, and biological-based remediation approaches. Bioremediation, where microorganisms are used to metabolize or transform pollutants, has appeared to have a favorable result for both PAHs and pesticides. The biodegradation of PAHs can be achieved by some fungi and bacteria, which transform the carcinogenic OPs into less toxic forms. Hence, such an eco-friendly approach is crucial for reducing environmental contamination and minimizing the contamination of humans with carcinogens (Barathi *et al.* 2023). Phytoremediation, applying plants to detoxify and eliminate pollutants, also results in an eco-friendly manner to manage pesticide-contaminated soils (Soudani *et al.* 2022).

2.3. Emerging contaminants

Apart from the most commonly occurring environmental contaminants, contaminants such as microplastics and pharmaceuticals are emerging contaminants that cause significant problems to the environment and human health globally (de Jesus *et al.* 2023). Pharmaceuticals refer to several medical compounds, including hormones, antibiotics, and antidepressants, which mainly originated from drug-producing companies and various health care settings. They are increasingly found in water bodies because of partial removal during wastewater treatment processes (Daughton & Ternes 1999). These biological wastes can contaminate the aquatic ecosystem and dramatically affect aquatic life and human health. For instance, the contamination of public environments with antibiotics can cause the development of antibiotic-resistant bacteria in the environment, which will be disseminated against humans and cause a significant public health problem (Kümmerer 2009).

Microplastics, considered as plastic particles below 5 mm in size, have become omnipresent in freshwater and marine systems (Osman *et al.* 2023). These tiny particles come from different sources, including the decomposition of larger plastic debris, microbeads in personal protective materials, and synthetic fibers from clothing (Thompson *et al.* 2004). Microplastics can be assimilated by a wide range of marine organisms, from whales to plankton, causing physical and chemical impact. Ingested microplastics bring a reduction in feeding efficiency, internal injuries, and transfer toxic chemicals to organisms (Galloway *et al.* 2017). In addition, microplastics can serve as carriers for pollutants such as HMs and OPs, which maximize their negative impact on the environment.

The management of environmental pollution is a multi-stage process that requires a collaborative effort from different sectors. For instance, the treatment of wastewater using oxidation systems or membrane bioreactors has demonstrated success in cleaning water from toxicants such as pharmaceutical waste (Luo *et al.* 2014). In addition, some rules and guidelines that are key agents to minimize the use of plastics and encourage proper disposal also curb the disposal of microplastics into the environment. A deeper investigation is needed to precisely outline the mechanisms, occurrences, and influences of emerging pollutants to unveil effective approaches to dealing with the issue and eradicating it.

3. Remediation and Types of Remediation

Environmental remediation is one of the key tools for the restoration and maintenance of the natural components of the ecosystem. The rapid increase in urbanization, industrialization, and agricultural intensification has contributed to a rise in water, air, and soil pollution, constituting substantial hazards to public health, biodiversity, and natural systems (Bediako *et al.* 2023). Due to the magnitude and sophistication of environmental contamination, multidisciplinary solutions are crucial. Usually, environmental remediation strategies are centered on HMs, pesticides, and industrial chemicals. Nevertheless, the introduction of new contaminants, such as microplastics, pharmaceuticals, and per- and polyfluoroalkyl substances, has demonstrated the imperative for innovative strategies to generate efficient and timely solutions (Bediako *et al.* 2023).

Remediation methods can be categorized into chemical, physical, and biological techniques. Physical remediation approach involves various physical-based activities, and it is mainly employed via skimmers, sorbent materials, and booms. A boom acts as a physical barrier, absorbing oil pollutants and preventing their dissemination until efficient additional remediation activity can be implemented (Vocciante *et al.* 2024). Following the application of boom, skimmers, and sorbents are then implemented to grip pollutants at the point of pollution (Dhaka and Chattopadhyay 2021). One critical challenge in the application of boom is that it depends on buoyancy and roll response. Buoyancy facilitates the floatation of the boom on the water's surface, while roll response correlates to the torque vital to rotate the boom from its vertical position. A better roll response boosts the success of the remediation process (Md Anawar & Chowdhury 2020).

Chemical remediation employs various substances, including clay minerals, phosphate, biochar, aluminum salts, silicocalcium materials, and sulfides, to stabilize and remove heavy metals from the environment. These chemicals operate through processes such as adsorption, reduction, oxidation, complexation, precipitation, and ion exchange (Md Anawar & Chowdhury 2020). While chemical remediation is efficient and straightforward, it is essential to consider that the chemicals used can also pose a risk of becoming pollutants themselves.

Despite the simplicity and the flexibility of chemical remediation, the chemicals utilized in such a type of activity can become pollutants to the environment (Md Anawar & Chowdhury 2020). In contrast, bioremediation offers a sustainable, cost-effective, and safe approach to pollution treatment (Patel *et al.* 2022). Such an approach can utilize microbes, plants, and their products where their activity mainly depends on the extent of contamination coupled with the type and location of pollution (Patel *et al.* 2022). Microbial agents are known for their positive characteristics, which make them advantageous over plants as they are simple to handle in the lab and have a quick growth in cultivation.

Microbial agents such as fungi and bacteria are naturally occurring biodegrading agents known worldwide (Das & Dash 2014). According to literature, microbes serve as a bioremediation tool in two ways—i.e., via the mobilization (conversion to a non-toxic form) and immobilization (total elimination) system (Verma & Kuila 2019). Activities such as bioleaching, bio-stimulation, enzymatic oxidation, and bioaugmentation are considered as mobilization types. While precipitation of the pollutant, biosorption, and bioaccumulation are considered as immobilization methods (Ayangbenro 2019; Bouabidi *et al.* 2019). This bioremediation approach is extremely crucial for decontaminating heavy metals in heavily contaminated sites.

4. General Overview of Bioremediation

Bioremediation is an environmentally friendly, promising, and cost-effective approach for transforming toxic pollutants into less harmful substances (Sonune 2021). Biological remediation can be implemented in any contaminated area using naturally occurring or GMOs and plants; nevertheless, plants obviously need a long time to grow and are unmanageable in comparison to the small-sized microorganisms. This implies microorganisms are a more attractive choice in the process of bioremediation. Furthermore, microorganisms are productive in decreasing the levels of HMs contamination and maximizing soil fertility and plant growth (Mishra *et al.* 2017).

There are three commonly employed decontamination methods known globally, and these are physical, chemical, and biological remediation. Physicochemical techniques are less recommended due to their minimal decontamination capacity of environmental waste and pollution (Rebello *et al.* 2021). Bioremediation offers a cost-effective and practical way of avoiding environmental contaminants. Investigations in this field are chiefly concerned with bacterial practices, which give various ranges of applications. However, archaea also play a role in bioremediation in many situations where bacteria are applied (Kour *et al.* 2021).

Bioremediation can be carried out either directly at the site of the actual contamination that occurred—called an on-site application (e.g., washing)—or by the process called off-site application, whereby contaminated materials are transported to a different location employing specialized facilities and without disturbing the biotic and abiotic ecosystem.

5. CRISPR Technology

5.1. Overview of CRISPR technology

CRISPR technology is one of the recently discovered genome editing technologies with various advantages over conventional tools. This technology was first identified in the immune system of bacterial pathogens, which they used to evade the viral attack by degrading its genome during the infection (Modell *et al.* 2017).

Currently, the CRISPR-Cas gene editing system is widely regarded as an efficient and productive tool (Alqahtani *et al.* 2024). Three types have been identified as I, II, and III (Alqahtani *et al.* 2024), and there are several subtypes that have existed (Alqahtani *et al.* 2024). Each Cas type is correlated to a specific Cas protein and the Cas9 DNA endonuclease. For instance, it uses RNA guidance to target and disrupt foreign DNA (Jiang & Doudna 2017). This is through the harnessing of this natural mechanism that experts have advanced a powerful tool that can be used to edit the genomes of multiple different organisms, including plants, animals, and humans. Unlike numerous other systems, CRISPR-Cas9 relies on a guide RNA (gRNA) to direct the Cas9 enzyme to a specific sequence in DNA and introduce a cut. This targeted DNA cleavage enables researchers to adjust genomic pieces (delete, add, etc.) at accurate loci in the genome, making this tool powerful and an invaluable attempt for biotechnology, scientific research, and potential medical applications (Yang *et al.* 2020).

The advancement of genomic research is closely linked to CRISPR technology as it simplifies the complexity and enhances the efficiency of the zinc-finger nucleases and transcription activator-like effector nucleases dependent approaches (Beumer *et al.* 2013). CRISPR technology maximizes the efficacy and precision of genome editing with its favorable properties, enabling the technology to generate significant results in various sectors, including agriculture and human health, by strengthening food security and the sustainability of the environment and by curing diseases associated with genetic disorders, respectively (Kuiken *et al.* 2021; Das *et al.* 2022).

CRISPR technology can be employed to provide lifelong treatment in humans. The application of CRISPR technology to cure beta-thalassemia and sickle cell disease by replacing the diseased cells with healthy blood cells is one crucial example that amplifies the power of the CRISPR technology in human medicine (Demirci *et al.* 2019). Besides, this technology can be employed in personalized medicine, thus maximizing the clinical intervention of various diseases (Selvakumar *et al.* 2022).

Regardless of its transformative ability, CRISPR technology also raises ethical and safety issues. The occurrence of off-target effects, where mistargeted editing of the genome, poses a potential health risk (Tsai & Joung 2016). Scientists are constantly improving the technology to improve its specificity and reduce these off-target defects. Ethical debates emphasize the application of CRISPR for editing of the germline, where the transformed DNA can be inherited and passed on to the coming generations as a long-term effect (Lanphier *et al.* 2015). Upon the advancement of CRISPR technology, robust ethical platforms and regulatory guidelines would be crucial to maintain its multidimensional uses (Zhang *et al.* 2020).

5.2. Historical progress

In 1987, a scientist discovered the very first CRISPR array in the *E. coli* genome as several series of regularly spaced repetitive sequences, whose functions could not be understood then (Ishino *et al.* 2018). Thereafter, bioinformatics investigations provide insights into the physiology of the CRISPR-Cas systems (Makarova *et al.* 2011). One study described conserved operons that seemingly encoded a novel mechanism of DNA repair, now known as Cas genes, and another CRISPR-correlated arrays to these Cas genes (Jansen *et al.* 2002). So, the documented reports demonstrated that the spacer nucleotide sequences within the CRISPR arrays were found to be closely related to the phage genomes, implicating the presence of a viable correlation between the CRISPR system and phage immunity (Pourcel *et al.* 2005). It was noticed that in *Streptococcus thermophilus*, studies showed that it had more spacers that line up with phage sequences and a large CRISPR-associated protein that has a DNA-cleaving HNH domain, which has been named Cas9 and is one of the proteins necessary for type II systems (Bolotin *et al.* 2005). Nonetheless, the knowledge of the specific function of CRISPR spacers in immunity remained uncertain.

A recent investigation on the type II CRISPR-Cas system in *S. thermophilus* shed light on the fact that there are spacers within the CRISPR array that are derived from phages, which assist grant immunity with those specific phages that have matching sequences. In addition, Cas genes are fundamental for the development of immunity as well as neutralizing the viral attack linked to phage infection (Barrangou *et al.* 2007).

The researchers revealed that CRISPR-Cas serves as an adaptive immune system in microbes. To gain a deeper insight into the functioning of the immune system, CRISPR-Cas, researchers have been studying type I systems, whose results demonstrated that the CRISPR array is transcribed and processed into short CRISPR RNAs (crRNAs) that identify and target a specific phage that attacks the DNA of the bacteria (Brouns *et al.* 2008).

5.3. Mechanisms of Action

The immune defense mechanism of bacterial cells towards the nucleic acid of viral infectious agents was the reason for the discovery of the CRISPR-Cas system. This adaptive process is initiated at the acquisition stage, where the foreign genomic segment is introduced into the CRISPR of the infected host. The distal sequences of the DNA known as spacers act as a huge section of adaptive immunity for the hosts, reminiscent of past infections (Barrangou *et al.* 2007). In the event of subsequent infections, the newly formed CRISPR array happens to appear as a precursor CRISPR RNA (pre-crRNA), which, in turn, becomes a mature form of CRISPR RNA (crRNA) after postprocessing. The crRNAs lead the Cas proteins to the viral DNA, where the effector complex cuts the invasion of the genetic material, and hence, the pathogen is neutralized (Brouns *et al.* 2008).

The CRISPR system that draws the most attention for gene manipulation is the identification of type II CRISPR-Cas9 that comes from *Streptococcus pyogenes*. Cas9 introduces a direct cut into the target genome at a specific location using crRNA and trans-acting crRNA containing single-guide RNA (sgRNA). After that, the host-derived non-homologous end joining (NHEJ) and homology-directed repair (HDR) can be utilized to repair the double-strand breaks (Jinek *et al.* 2012). NHEJ is repaired through direct insertion or deletion, which can affect the gene function, which is helpful in creating knockout models. On the other side, HDR involves a step that uses the base pair of the donor template to repair the break (mutation correction) (Doudna 2014).

CRISPR-Cas9 is one of the emerging systems of this type due to its use in genetic engineering in different types of organisms, starting from their smallest forms such as microorganisms, to the biggest ones such as plants and animals. The straightforwardness, productivity, and accuracy of the CRISPR-Cas9 system have empowered its utilization in areas such as functional genomics, drug design, and biotechnology. For example, CRISPR technology has been harnessed for the development of disease models, driving genetic solutions to crops and rectifying the defective genes that turned into genetic diseases such as sickle cell anemia and cystic fibrosis (Hsu *et al.* 2014). Research is also underway to mitigate the negative consequences of this technology, which will make it not only more accurate but also reduce off-target effects and ensure its safe use in medical settings (Chapman *et al.* 2017).

5.4. Advantages

CRISPR-Cas9 is a superior molecular approach to the previous gene editing technologies, such as ZFNs and TALENs. Its primary advantage lies in its simplicity and ease of use. Unlike ZFNs and TALENs, which require precise construction for each target site, CRISPR-Cas9 works with an sgRNA that can be easily generated to target any region of the host genome (Doudna 2014). CRISPR has become a more reachable and effective tool for investigators (users) because of its simple design platform, lower processing cost, and efficiency as well.

Multiplexing (flexibility) is the other positive value of CRISPR technology, which opens the door to targeting multiple genes at once. This phenomenon is chiefly crucial when it comes to investigating polygenic traits and networked genetic interactions that involve multiple genes (Cong *et al.* 2013). Nevertheless, ZFNs and TALENs are constrained because they perform their editing activity via proteins, and this facilitates the process of editing multiple sequences in parallel, which is more difficult than CRISPR.

CRISPR also minimized the burden on researchers to identify multiple sgRNAs that can target different genomic loci simultaneously, and hence, simplify high-throughput genetic screens and expedite the functional genomics studies (Shalem *et al.* 2014).

CRISPR also has a higher efficiency of gene editing and a higher level of precision that can be attributed to its predecessors. Likewise, high specificity is because the sgRNA docks perfectly onto the target DNA sequence to recruit the Cas9 nuclease with precision at the site to make its cut (Jinek *et al.* 2012). The implementation of newer designs of sgRNA and better Cas9 protein has again reduced off-target effects, making the CRISPR system more precise (Hsu *et al.* 2014). On the contrary, ZFNs and TALENs demonstrate lower targeting efficiency and more frequent off-target cleavage, which collides with safety considerations and can trigger adverse genetic modifications (Kim & Kim 2014). All these positive characteristics of CRISPR make it superior to the conventional tools for genetic engineering and therapeutic use.

5.5. CRISPR-Enhanced Bioremediation

CRISPR technology can be employed for the remediation of the polluted environment by generating genetically modified microbes and plants, as displayed below (Figure 2).

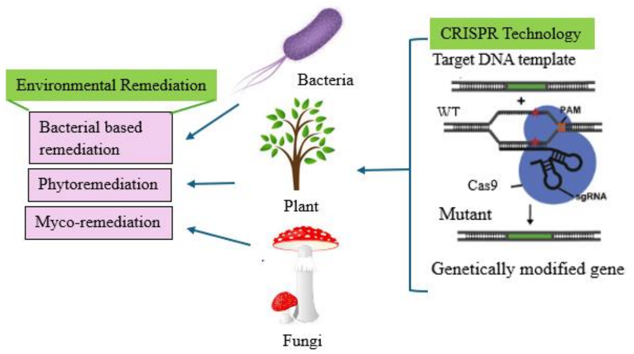

Figure 2. Schematic diagram showing the approaches of CRISPR technology for environmental bioremediation

5.5.1. Microbial Bioremediation

(i) Bacterial Bioremediation

CRISPR-Cas9 has presented novel prospects in the area of microbial-based bioremediation since this technology introduced medically relevant modifications to the degradation potential of a diverse array of toxic contaminants. In the past, the bioremediation process employed either naturally occurring or conventionally developed microorganisms to degrade the pollutants in the environment. By using CRISPR, researchers can develop specific mutations that would improve the microbes' capabilities of breaking down toxic substances – HMs, hydrocarbons, and a diverse array of industrial chemicals (Yao *et al.* 2018). For instance, it has been employed to increase the activity of certain enzymes related to degradation processes and promote the rate of pollutants' degradation (Singh *et al.* 2021).

One more crucial beneficial effect of employing CRISPR in microbial bioremediation is the creation of a microbial community with different potential and remediation characteristics (Singh *et al.* 2021). Manipulation of various microbial species allows obtaining consortia where every member would be optimized for breaking up certain types of pollutants and complex toxic contaminants. The significance of this approach is not limited to the entire efficacy of the bioremediation processes but is also crucial in maintaining microbial balance in the ecosystem. In addition, CRISPR technology can optimize the metabolic potential of the microbial agent, thereby enhancing the pH tolerance or resistance to toxic by-products (Fokum *et al.* 2019).

Currently, CRISPR-assisted bioremediation displays the opportunity for in situ monitoring and control of microbial activities at the spot of contaminated sites. By harnessing the biosensor technology into the CRISPR system, researchers could develop an innovative platform that not only degrades pollutants but also alerts when the degradation has taken place or if further intervention is required. Such an organized approach is unparalleled and guarantees the safety and efficacy of the process of bioremediation, and minimizes the risk of unanticipated environmental damage. Upon the advancement of this technology, CRISPR is projected to play an innovative role in the maturity of efficient, eco-friendly, and sustainable bioremediation strategies (Sahoo *et al.* 2022).

Escherichia coli is one notable example of the modified microbial strain that has been engineered for bioremediation activity to capture heavy metals such as cobalt and nickel. The Ni3Co strain 'NiCo Buster' was developed to successfully accumulate these metals, proving them to have high potential in bioremediation within contaminated environments (Tarek & Ali 2022). Similarly, *Cupriavidus metallidurans* was genetically modified to enhance its resistance and ability to detoxify mercury (Tarek & Ali 2022). Recent research findings indicate that CRISPR-modified *Pseudomonas putida* showed its ability to decompose aromatic hydrocarbons and related industrial pollutants. These modifications appear to be beneficial in metabolizing hazardous chemicals (Sahoo *et al.* 2022).

In a different investigation, researchers engineered *Bacillus cereus* to display genes that degrade mercury (Hg) and biosorb for the deterioration of Hg in polluted water (Tarek and Ali 2022). The results obtained in such studies served as evidence for the potential of CRISPR-based bioremediations.

(ii) CRISPR in Myco-remediation

The use of CRISPR technology in myco-remediation has been an emerging bioremediation approach, which enabled us to modify fungi's genomes and opened the door to maximizing the potential of microbial-based bioremediation for decomposing complex pollutants. CRISPR/Cas9 enables researchers to modify the genomic organization of fungi to express genes that are responsible for efficient and safe degradation of toxic compounds, thereby protecting the environment (Shanmugam *et al.* 2019). According to documented findings, genetically engineered fungal strains are designed to generate higher levels of enzymes that degrade organic pollutants such as lignin, making myco-remediation a more crucial and promising approach for the cleanup of the environment (Kumar & Dwivedi 2019).

CRISPR technology boosts the potential of fungal enzyme production which is playing the leading role in environmental pollutants degradation. Some of these enzymes such as peroxidases, laccases, and cellulases, are known for their biodegradation of organic pollutants of the ecosystem. Today, researchers have produced metabolically superior fungal strains using CRISPR, thereby optimizing the bioremediation process (Harms *et al.* 2011).

Fungi generated by using CRISPR technology are usually utilized for the decontamination of soil and in water. In the process of soil remediation, such types of fungi can be applied to the spot of contamination, thereby enhancing the soil health and protecting against environmental degradation (Harms *et al.* 2011). Similarly, in water remediation, modified fungi can be employed to clean up wastewater and contaminated bodies of water by decomposing pollutants that will ultimately lead to a cleaner biosphere. The broad diversity of fungi, coupled with the advancement of CRISPR technology, makes myco-remediation a promising solution for tackling simple as well as complex environmental pollution.

The hyphae of fungi can be dispersed through soil, which resists high levels of pollutants and eliminates contaminants through the process of enzymatic activities and physical uptake. Recent progress in CRISPR/Cas9 technology allows researchers to introduce targeted genetic manipulation and augmentation of xenobiotic metabolism, which can serve as a powerful strategy to generate genetically modified fungi for biological cleanup of toxic compounds (Stein *et al.* 2018).

5.5.2. CRISPR in Phytoremediation

Conventional phytoremediation suffered from some limitations linked to the natural characteristics of the plant and related features. Unlike conventional phytoremediation, CRISPR technology has significantly advanced the area of phytoremediation by improving the capability of plants to depollute pollutants from the diverse array of ecosystems (Nayeri *et al.* 2023). CRISPR technology strengthens the biodegradation capacity of plants by introducing special characteristics that are linked to pollutant uptake and detoxification. As an example, scientists can introduce or enhance genetic material that codes for proteins responsible for metal transport and storage within the tissue of plants, thereby improving their capacity to absorb and neutralize toxicants such as Cd, Pb, and with lower cost and time needed (Patra *et al.* 2020).

Upon the use of CRISPR-Cas9 technology, the bioactive compounds produced by the engineered plants can be boosted. Such modifications ultimately result in tolerance to pollutants and quicken the denotification process of significant pollutants from the ecosystem (Naz *et al.* 2022).

By CRISPR technology, one can generate enhanced root exudate and facilitate the phytoremediation process. Root exudates are synthesized by the root of a plant, which is used to attract and sustain beneficial soil microbes. Microbial communities attracted to the polluted area facilitate the detoxification and clearance process and optimize the overall efficiency of the phytoremediation process (Sharma *et al.* 2023). According to Banerjee, CRISPR modified *Arabidopsis thaliana* has been shown to be efficient in accumulation and tolerance to arsenic pollution (Banerjee and Roychoudhury 2019). Table (1) summarizes some documented research findings associated with the impact of CRISPR technology in bioremediation.

6. Environmental and Safety Considerations

The deployment of organisms via the application of the CRISPR system presents serious biosafety issues. New breed GMOs, ones made by CRISPR technology, pose a danger to the surrounding ecosystems (Movahedi *et al.* 2023).

To deal with all these biosafety issues, containment and control measures are of paramount importance. These strategies include measures to protect the release of organisms that have undergone CRISPR editing. Some of the constraints linked to this technology can be minimized via implementing numerous containment interventions, including physical, genetic, and environmental monitoring (Ahmad *et al.* 2024). In addition, the experience shows that the stable regulation of measures in emergencies and the development of the corresponding plan, with the subsequent protection of the environment from possible accidental releases, is essential (Ahmad *et al.* 2021).

Table 1. Summary of research findings associated with the impact of CRISPR technology in bioremediation

Biological agents	CRISPR technology employed and effects	Reference
Acidithiobacillus ferridurans JAGS	Downregulating the transcriptional levels of genes involved in sulfur oxidation to avoid sulfidic minerals contamination	(Chen *et al.* 2023)
Deinococcus geothemalis	The addition of mer operon from *E. coli* to bacterium *Deinococcus geothemalis*. reduce the Hg pollution even at higher temperature	(Dixit *et al.* 2015)
Cupriavidus metallidurans	*Cupriavidus metallidurans* modified genetically with pTP6 plasmid considerably minimized the Hg from polluted soils	(Dixit *et al.* 2015)
Tobacco and *Arabidopsis* plants	CRISPR-mediated expression of genes (overexpression of metallothioneins encoding-genes (MT1, MT_{A1}, and MT2)) *Arabidopsis* and tobacco plants have improved their ability to withstand and accumulate Cd, Cu, and Zn	(Lv *et al.* 2013)
Hirschfeldia incana	Expression of the metallothionein gene, MT2b, tolerate and accumulate of Pb	(Auguy *et al.* 2016)
Brassica juncea	The transfer of the genes APS and SMT, responsible for the synthesis of ATP sulfurylase and selenocysteine methyltransferase, respectively, enhanced the tolerance and accumulation of Se in *B. juncea* plants.	(LeDuc *et al.* 2006)
Tobacco plants	The Overexpression of a plasma membrane protein (NtCBP4) enhanced the bioaccumulation Pb in transgenic tobacco plants.	(Arazi *et al.* 1999)
Arabidopsis and tobacco	The introduction of MerC genes in Tobacco and *Arabidopsis* maximize the accumulation of Hg by 2-fold	(Sasaki *et al.* 2006)
Alfalfa plants	Expression of the gene, BphC.B, by transgenic alfalfa plants, substantially risen their tolerance to Polychlorinated biphenyls and 2,4-dichlorophenol (2,4-DCP)	(Wang *et al.* 2015)
Rice (*Oryza sativa* L.)	The knocking out of the metal transporter gene, OsNramp5, result in the accumulation of Cd in rice.	(Tang *et al.* 2017)
Rice (*Oryza sativa* L.)	CRISPR/Cas9 Mutant Rice Ospmei12 result in Oryza sativa to resist Cd stress	(Li *et al.* 2022)

Despite the numerous benefits generated from CRISPR-modified microbial bioremediation, rules and regulations have become crucial in regulating the process. Different international/national organizations have formulated rules and regulations concerning the approval of genetic modifiers. For example, the United States' Environmental Protection Agency (EPA) and the European Food Safety Authority (EFSA) developed methodologies for the evaluation of risk posed by GMOs, including those developed through CRISPR technology (Hilbeck *et al.* 2020).

Due to the dynamic nature of CRISPR technology, rules and regulations need to be updated regularly to resolve new challenges and existing constraints as well. For instance, the Organization for Economic Cooperation and Development (OECD) has unveiled new guidance on the environmental safety of biotechnology-edited plants and microorganisms. These new guiding principles stress the assessment of varied and long-term risks and the active cooperation of different countries to navigate the opportunities of using CRISPR instruments (Strotmann *et al.* 2023).

7. Challenges and Future Prospects

CRISPR-based bioremediation still has several technical constraints to deal with concerning the advancement of the method. However, one main issue is the singularity of CRISPR agents, which is their introduction to the desired microorganisms within environmental conditions. The smart delivery systems in heterogeneous environments, including contaminated soil and water bodies, remain imperative for their technological advancements and applications as well (Zhu 2022). Also, the success of CRISPR systems in a wide range of ambient environments, such as the fluctuation of environmental temperature or pH, is still a challenge (Lino *et al.* 2018).

One of the genomic complications that can be linked to the use of CRISPR-based bioremediation is the phenomenon known as horizontal gene transfer (HGT) and its consequences. HGT is described as the movement of genetic material from one organism to another through a process that can occur through transformation, transduction, or conjugation (Watson *et al.* 2018). According to documented findings, genetically edited genes can be integrated into other organisms and become a cause of conflict with ecosystems or spread to the existing population (Brokowski, C. and Adli 2019). This constraint can be rectified by generating organized directions to minimize the frequency of gene transfer and its environmental adverse effects.

Advanced and next-generation CRISPR-mediated bioremediation is crucial for better specificity and fewer off-target effects (Barooah and Hazarika 2022). Besides, supporting or integrating CRISPR technology with other relevant techniques, including synthetic biology or the very advanced microbial engineering, can open new prospects for the improvement of bioremediation potentialities (Barooah & Hazarika 2022). Scientists also need to analyze how the changes attained with CRISPR stay long-term and are safe in nature.

New methods of genome editing, including epigenome editing based on CRISPR, provide the opportunity to optimize the process of bioremediation and expand the area of its applications (Barooah and Hazarika 2022). Moreover, the use of CRISPR in combination with computational predictions and systems biology might reduce the interspecies competition and improve tactics of bioremediation (Verdezoto-Prado *et al.* 2024). The viable collaboration of different disciplines will help to resolve the difficulties that have been viewed today and are expected to appear in the future.

8. Conclusion

CRISPR technology in bioremediation offers great potential to treat the cardinal problem of environmental pollution. Despite ethical issues, regulatory barriers, and possible environmental effects, this technology can be integrated with other interdisciplinary biotechnological strategies and contribute a lot to the remediation of contaminated and polluted environments. While it is true that CRISPR technology has various potential, considering the particular engineering of microbes for targeted pollutant degradation, translation will need to overcome severe ecological and regulatory challenges. Definitely, the alleviation of hazards related to uncontrolled gene transfer and long-term safety of engineered organisms in nature has to be selected through structured governance and careful research. The development of next-generation CRISPR tools is required, such as epigenome editing, and an crucial interdisciplinary collaboration with synthetic biology and computational predictions. Finally, improvement of accuracy and long-term stability, with responsible integration of CRISPR, is needed to release a safe, sustainable, and powerful strategy to the large-scale restoration of environments and pollution control globally. Hence, future research shall focus on creating and evaluating new GM microorganisms to cope with the current and future challenges of environmental pollution.

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