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Mechanistic Approach of CRISPR-Cas Technique in Mycoremediation of Polycyclic Aromatic Hydrocarbons

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are one of the most hazardous constant organic contaminants that consist of multiple aromatic rings arise from the incomplete burning of carbon-based material. When these toxic compounds exposed to the atmosphere, water and soil causes some major health issues to human and other living organisms. Therefore, it is necessary to remove from the environment. Although there are different technologies are introducing to remediate these hazardous pollutants but most of them faces challenges and has negative impact on environment except bioremediation technique. Mycoremediation is one of bioremediation technology where fungi are used for the remediation of pollutants. Nowadays integration of CRISPR/Cas9 technology in mycoremediation makes more beneficial for removal of pollutants from environment because it enhances the remediation efficiency. Till now many research has done in gene editing of fungi to enhance the degradations of pollutants. Although there are so many research is going on but still it is very difficult to understand the mechanistic behavior of CRISPR/Cas9 in fungal gene editing for improvement of mycoremediation. Therefore, in this review an overview on mechanism of CRISPR/Cas9 in fungal genome editing for degradation of PAHs and their regulatory pathways are discussed to better understand the mycoremediation of PAHs.

Keywords: Mycoremediation, Polycyclic aromatic hydrocarbons, Pollutants, CRISPR/Cas9, Fungi

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are hazardous persistent organic compounds that arise from the partial burning of fossil fuels, volcanic eruption and from natural hazards. In atmosphere they are found in two phases such as. The vapor phase and solid phase PAHs are expose into the organism from environment via breathing, eating food, smoking etc. The plants absorb PAHs from soil and water by roots and stomatal opening of leaves (Liu *et al.,* 2024). When they expose into the environment causes some negative impacts into the organisms. For example, if it enters into the plants cell then it decreases the growth, rate of metabolism and photosynthesis. Moreover, when PAHs are present in soil and water at high concentration then it causes some acute toxicity to aquatic and terrestrial organisms. Generally high molecular weight PAHs are known for causing cancer, reproductive abnormalities, development and immunity related problem in human health. Therefore, removal of PAHs from the environment is very crucial. Till now various technologies (biodegradation, photochemical degradation, chemical oxidation, leaching, bioaccumulation, etc.) have been used to remove PAHs from the environment. But all these mentioned technologies are time taking and have negative impact in the environment and can cost a fortune. Therefore, today's peoples prefer the bioremediation techniques to remove PAHs due to their environmental sustainability and cost-effective nature.

Mycoremediation is one of the bioremediation techniques where fungi are used to degrades the pollutants from the environment. Paul Stamets first coined the term Mycoremediation. Till now so many fungi have been used to remediate the environmental pollutants. Mycoremediation is a cost effective and environmentally friendly method that used to eliminate pollutants from the soil and water (Navina *et al.,* 2024). Depends on the location mycoremediation are classifies as in situ and ex situ mycoremediation. When fungi used to remediate pollutants in their original contaminated site than it is called as in situ mycoremediation. And in ex situ mycoremediation the contaminated site is removed and fungi are used to treated the contaminated material in a controlled environment (Rehman *et al.,* 2018). Now a days fungi are used in in situ remediation of dyes, herbicides, pharmaceutical drugs, polycyclic aromatic

hydrocarbons and heavy metals etc. Fungi release some enzymes and biosurfactants that helps to remove the contaminants from environment (Dinakarkumar *et al.,* 2024). Different types of lignolytic enzymes, cytochrome p-450 monooxygenase, dioxygenase, dehydrogenase epoxide hydrolases and glutathione transferase are release by the fungi and which completely degrades the PAHs into CO_2 and essential nutrients in soil. The fungi from the basidiomycetes are particularly used in the mycoremediation because they can easily break the complex PAHs into simpler form by producing the laccases and peroxidases (Pande *et al.,* 2019). Apart from the basidiomycetes the fungi from trichocomaceae family are also used in mycoremediation. While mycoremediation is a less expensive, ecofriendly method but still it has some limitations in scalability, monitoring, slow process and effective only for the organic compounds (Akhtar & Mannan, 2020). Mostly mycoremediation are used in laboratory scale but there are a very few applications are available in real field experiment because of the environmental factors and interaction between inoculated fungi and indigenous microbes. In comparison to other techniques mycoremediation is slow process (Zaman *et al.,* 2023). Since, fungi needs more time to colonize and favorable temperature for well growth. The growth of fungi also fluctuates by the change of pH and moisture level. Therefore, genetically modified fungi are used in the mycoremediation to enhance the removal of pollutants. As they have some specific qualities such as target specific pollutants, take less time to remediate and they can symbiotically coordinate with other organisms to speed up the degradation process (Akpasi *et al.,* 2023).

CRISPR-Cas is a well-known genome editing technology that is used in the precise modifications of DNA sequences. From the previous study found that now a days CRISPR-Cas system is highly relevant to improving fungi for mycoremediation of pollutants. This system allows the target modification of fungal genes to enhance their ability to degrade environmental pollutants by producing some beneficial enzymes (Shanmugam *et al.,* 2019). This system was discovered by two legendry women Emmanuelle Charpentier and Jennifer Doudna. This system is also known as “genetic scissors” due to its functions as a cutting tool at the molecular level (Gostimskaya, 2022). CRISPR (clustered regularly interspaced short palindromic repeats) is a repeating DNA sequence of bacterial immune system and Cas 9 is a protein enzyme that acts as the molecular scissors (Mengstie & Wondimu, 2021). It is the main component in CRISPR/Cas9 which consist of more than 1000 amino acid residues also it is the only Cas protein that used in genome editing first. Cas9 protein was extracted from *Streptococcus pyrogenes* (SpCas-9). Recognition (REC) lobe and the nuclease (NUC) lobe are two regions of Cas9 (Xu & Li, 2020). REC1 and REC2 domains of REC lobe helps to bind guide RNA and RuvC, HNH, protspacer adjacent motif (PAM) interacting domains of NUC lobe used to cut each single-stranded DNA and PAM helps to bind the target DNA (Mengstie & Wondimu, 2021). In mycoremediation it helps to improve natural remediation capabilities of fungi leading to more efficient and detoxification of pollutants. They help to increase the production of metabolites and bioactive compounds in endophytic fungi by modifying different target genes (Ma *et al.,* 2025). Till now many researches are ongoing on CRISPR/Cas technology in mycoremediation but no significant review is done on this topic to the best of our knowledge. Therefore, in the present review broadly explained on the mechanism of CRISPR/Cas9 technology in genome editing to better understand how fungi improve the degradation of PAHs.

CHALLENGES IN BIODEGRADATION OF PAHs

PAHs are common persistent pollutants of soil and water nowadays. Due to contamination of PAHs in soil breaks the physicochemical bonding of soil which reduces the water holding capacity and make the soil unfavourable for the agricultural practices. Although different technologies are used to remediate PAHs from the environments but they have some negative impacts in the environments and very expensive (Gundlapalli *et al.,* 2024). However, mycoremediation is a technique of bioremediation which is economically feasible and ecofriendly by nature that can be used in the degradation of PAHs. Till now many indigenous and genetically engineered fungi has been used in the degradation of PAHs from soil and water (Bala *et al.,* 2022). There are some challenges faces in the degradation of PAHs from the environments (Figure1). For example, PAHs are hydrophobic in nature therefore, they tend to adsorb onto soil and

sediment particles, reducing their accessibility to degrading microorganisms (Tang *et al.,* 2025). Since, the PAHs are consist of multiple aromatic rings so they are chemically stable in nature and resistant to biochemical attack. Also, after degradation of PAHs they can form some secondary pollutants which could be more dangerous for the living organism (Patel *et al.,* 2020). If PAHs are contaminated with heavy metals, then it not only degrades the soil fertility but it also degrades the diversity of soil microflora. Moreover, the natural degradation of PAHs is a very slow process therefore to increase the time of degradation some genetically engineered microbes are used (Ali *et al.,* 2022).

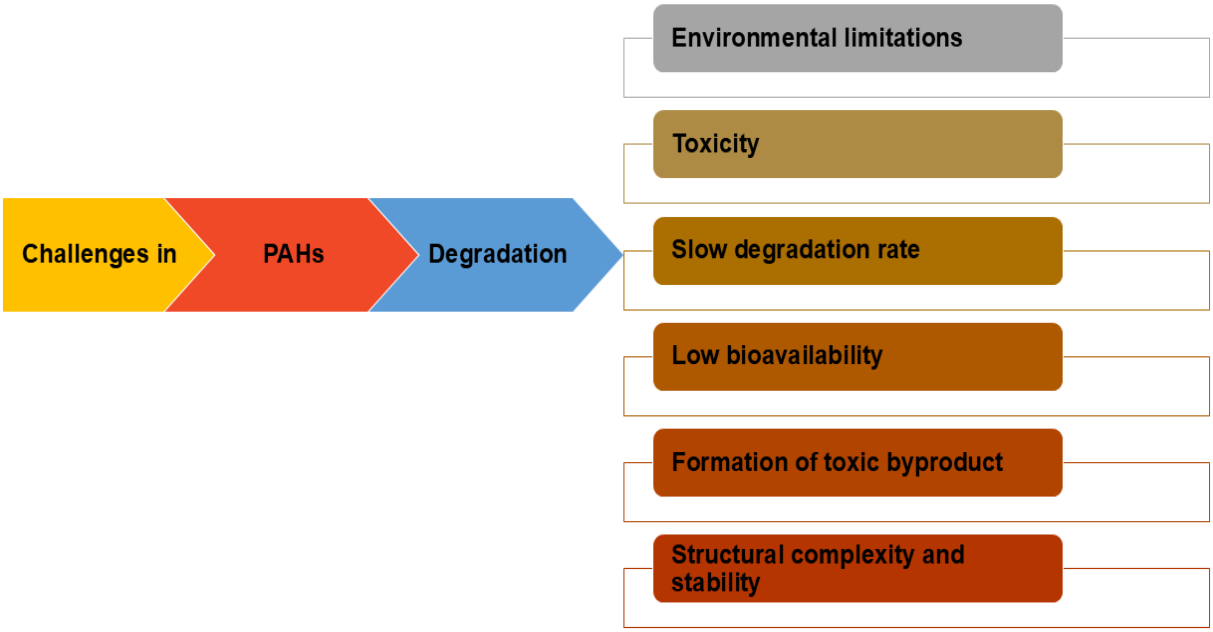

Figure 1: Some challenges face in PAHs degradation

MYCOREMEDIATION MECHANISM OF PAHS

Fungi is one of the very opportunistic, quickly response, and widely adaptable microbes that can easily breaks the toxic PAHs rings into simpler form by releasing the catalysing enzymes (Dinakarkumar *et al.,* 2024). Oxygenase, dehydrogenase, laccase, lignin and manganese peroxidase, lignolytic enzymes are some significant class of enzymes that plays a very crucial rule PAHs degradation. They help to catalyze the molecular bonds of PAHs and transform into simpler metabolites through mineralization (Haritash & Kaushik, 2009). These enzymes are mainly produced by lignolytic (also known as white rot fungi) and non lignolytic fungi. They oxidise the PAHs to biphenolic intermediates to form secondary metabolites of plants such as quinones. The fungi such as *P. chrysosporium* secret lignin peroxidase and manganese peroxidase enzyme which help to catalyze the anthracene, benzo[a]pyrene, pyrene, fluorene into quinone and anthraquinone. Later anthraquinone oxidised to form phthalic acid and carbon dioxide (Kadri *et al.,* 2017) (Figure 2). Fungal enzymes offer a sustainable and eco-friendly approach to bioremediation, as they can degrade pollutants without producing harmful byproducts. However, the mycoremediation faces some challenges due to environmental conditions, slower degradation rates, incomplete degradation, enzyme inhibition and scalability issues and competition with native microbes. Fungi are sensitive to salinity, pH, temperature so to grow faster and remediate a large number of pollutants from the environments it needs a favourable environmental condition. Fluctuations in environmental conditions reduces the effectiveness of breaking down pollutants (Dasgupta *et al.,* 2024). The fungal enzyme produce is faster, cost effective, scalable and amenable to genetic manipulations. Direct application of these fungi for the in-situ bioremediation of PAHs has several limitations, such as insufficient biomass growth and lack of established methodologies.

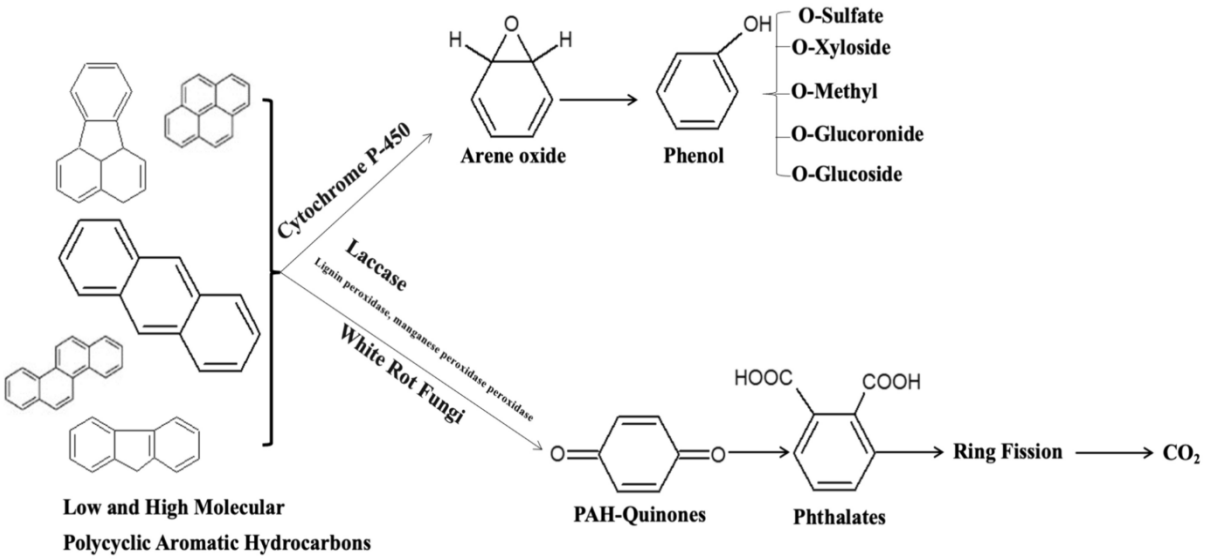

Figure 2: Mechanism of PAHs degradation by the fungi. © Reprints from (Omoni *et al.,* 2024)

IMPRESSION OF CRISPR/CAS9

CRISPR/Cas9 technology are divided into two class based on their appearance and functions of Cas-protein. They are again classified in some sub classes such as type I, III, and IV which contains multiple subunits of Cas protein. Second class of CRISPR/Cas again subdivided into type II, V and VI which consist of a single Cas protein (Ali *et al.,* 2022). Type II CRISPR/Cas technology mostly used in genetic engineering due to its simpler structure. Another two essential components of CRISPR/Cas-9 system are guide RNA (gRNA) and Cas-9 protein. In this technology a natural bacterial defence mechanism is used to precisely target and modify the double stranded DNA (Mengstie & Wondimu, 2021). The whole process is regulated by a guide RNA (gRNA) that commands a Cas protein (like Cas9 or Cas12a) to form a unique DNA sequence. After that Cas protein breaks the DNA double strand and renature with a desired DNA sequence insertion. A short guide (gRNA) is synthesized with a Cas protein (Cas9 and Cas12a) to match a specific DNA sequence in the target genome (Redman *et al.,* 2016). Once the gRNA-Cas protein complex is bound to the target DNA, the Cas protein acts as molecular scissors that cut the sequence in the targeted location (Aljabali *et al.,* 2024).

HOW CRISPR/CAS9 TECHNOLOGY ENHANCE MYCOREMEDIATION

By modification of genomes associated in enzyme production or secretion pathways, CRISPR/Cas9 can raise the efficiency and yield of desired enzymes. This can be achieved by enhancing the expression of existing enzyme-encoding genes or by introducing new metabolic pathways for enzyme production. Fungi have complex gene regulatory networks controlling enzyme production. It can be used to edit transcription factors or other regulatory elements that influence enzyme secretion (Pal *et al.,* 2024). For example, CRISPR/Cas9 technology is used to delete ACE1 gene in *Rhizomucor emersonii* to upregulate the XlnR, which increase the productivity of enzyme cellulase (Singh *et al.,* 2023). Similarly, the new genomee editing tool can also be used in the synthesis of transcription factor such as XlnR and GaaR that increases the production of hemicellulose and pectin in *Aspergillus niger*. This technology not only helps in enzyme secretion but also involve in protein secretion, such as those encoding components of the endoplasmic reticulum (ER) or golgi apparatus, potentially improving the efficiency of enzyme export. Moreover, except enhancing the secretion of enzyme and protein they can also improving or modifying the enzymes properties, viz. their substrate specificity, activity and stability. In endophytic fungi CRISPR/cas9 is used to change some specific genes for the improvement of plant growth promoting property and develop more advantageous endophytes (Verma *et al.,* 2023). Some examples of fungi where CRISPR/Cas9 technology is utilized to enhance the productivity of enzyme are given in **Table 1**

Table 1: Fungi where CRISPR/Cas9 technology used to enhance the enzyme production

Name of fungi	Secret enzyme	Edited gene	Reference
Aspergillus niger	Cellulase, pectinase	XlnR and GaaR	Kun *et al.,* 2020
Aspergillus oryzae	Catalase, esterase, sucrase	ACE1, XlnR	Katayama *et al.,* 2016
Rasamsonia emersonii	Hemicellulases, β-glucosidase A_GH3, endoglucanases, α-mannosidase_GH47	-	Singh *et al.,* 2023
Trichoderma reesei	Cellulase	-	Pant *et al.,* 2022
Penicillium subrubescens	Cellulases, xylanases	ku70	Salazar-Cerezo *et al.,* 2020
Rasamsonia emersonii	Cellulase, hemicellulase	ACE1	Singh *et al.,* 2023
Aspergillus fumigatus	Endoglucanase	*eglA*	Benites-Pariente *et al.,* 2024
Neurospora crassa	Cellulase	clr-2	Grüttner & Kempken, 2024
Myceliophthora thermophila	Cellulase	amdS	Liu *et al.,* 2017
Cerrena unicolor	Laccase, peroxidase	-	Zhang *et al.,* 2022

MECHANISTIC STUDY OF CRISPR/CAS9 IN MYCOREMEDIATION

CRISPR/Cas9 genome editing technology works in three different steps and they are recognition, cleavage and repair (Figure 3). The sgRNA commands Cas9 protein to recognise the target sequence of the interested gene by the help of 5′crRNA complementary base pair component (Du *et al.,* 2023; Mengstie & Wondimu, 2021). If the sgRNA is absent then the Cas9 protein remains inactive. The Cas-8 nuclease breaks the double strand of DNA molecule at a site of 3 base pair upstream to PAM which is a short (2-5 base-pair length) conserved DNA sequence downstream. This helps to the cut site of desired DNA and the PAM size is varies depending on the bacterial species (Manghwar *et al.,* 2019). Cas-9 protein is most commonly used genome editing enzyme and it helps to recognize the PAM sequence at 5′-NGG-3′ (N can be any nucleotide base). When Cas9 protein get the target site in PAM then it instigates the local DNA molecule to form RNA-DNA hybrid and later Cas9 protein start to cleave the DNA (Hassan & Yesmin, 2019).

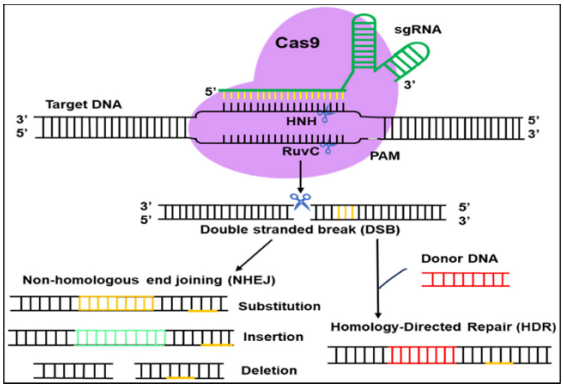

Figure 3: Mechanism of CRISPR/Cas9 © reprints from (Ma *et al.,* 2025)

POTENTIAL GENE TARGETS FOR ENHANCED PAHs DEGRADATION

As mentioned above PAHs degrades by fungi secreting some specific enzymes and CRISPR/Cas9 genome editing technology helps to improve the expression of these enzymes by modifying the targeted genes. Although CRISPR/Cas9 directly do not degrades PAHs from soil but they help to enhance the production of PAHs degrading agents by modifying genes associated in microbial degradation and phytoremediation (Naeem *et al.,* 2022). For example, cytochromes P450, laccase,

dehydrogenase enzymes are well known for having the PAHs degrading capacity, and these enzymes are secreted by some specific fungi. This specific fungus has a gene which are responsible for secretion of these enzymes and CRISPR/Cas9 technology works on those gene to increase the stimulation of enzymes (Arun *et al.,* 2023). Such as in filamentous fungi *Aspergillus niger* has a glucoamylase landing sites (GLSs) which secret abundant of protein due to presence of promoter and terminator site of glucoamylase gene (glaA) (Fiedler *et al.,* 2018). In this GLSs site on new DNA sequence known as dubbed KORE sequences is introduces by using CRISPR/Cas9 technology to upsurge the more protein productivity (Arentshorst *et al.,* 2023). Similarly, in *Cerrena unicolor* BBP9 the CRISPR/Cas9 technology were applied to regulate the production of laccase and manganese dependent peroxidase enzyme by disrupting the LacA and mnp3 genes (Zhang *et al.,* 2022). Moreover, they can also be used to enhance detoxification enzymes like epoxide hydrolases, dehydrogenases and transferases by precisely editing the gens that encode these enzymes, potentially leading to increased detoxification capacity in organisms exposed to harmful toxins. CRISPR/Cas9 target the gene encode the detoxification enzymes to break and repair with a desired genes which increase the production of enzymes (Geng *et al.,* 2024).

Mycoremediation of PAHs is tightly regulated by some pathways such as transcription factor, promoters and enhancers, signal transduction cascades. These pathways are controlled by induction of lignolytic enzymes (laccases, manganese peroxidase and lignin peroxidases), oxidative stress response, nutrient sensing (Kumar & Chandra, 2020). By applying CRISPR/Cas9 technology regulatory pathway in fungi can be enhanced for PAHs degradation. Some transcriptional repressor downregulates the expression of PAH-degrading enzymes under non inducing conditions such as CRISPR knockout strategy (Ansori *et al.,* 2023). In this strategy the repressor gene such as CreA is knock out to improve the expression of PAHs degrading genes with increasing nutrient availability. Next, by using this technology disrupting repressors or overexpressing transcriptional activators using CRISPR/Cas9 allows for enhanced and constitutive expression of catabolic pathways, increasing degradation efficiency (Sathee *et al.,* 2022). Next, it activates the transcription factors like Xyr1, Yap1, PacC that positively influence enzyme production or stress responses. This technology not only stimulate the fungi for pollutants degradation but also, they manipulate the genes involved in ATP synthesis and energy metabolism. These genes can be engineered to improve the fungal cell ability to generate energy, which is crucial for detoxification process and overall survival under stress condition caused by pollutants (Hara & Kondo, 2015).

MECHANISTIC INSIGHTS FROM OMICS AND SYSTEMS BIOLOGY

From the previous studies reported that growth of fungi decreases when contaminated with PAHs because they cause toxicity to fungal cells, interference with membrane properties and potential DNA damage. But in case of filamentous fungi during the stress of PAHs they produce cytochrome P450 and epoxide hydrolases that transform the PAHs into epoxide and oxidative product formation. Later six alkane-inducible cytochrome P450 and monooxygenase converts them into alkanes to fatty acids (Gao *et al.,* 2019). After breakdown of polyaromatic rings the resulting molecules enter into a pathway like beta-ketoadipate pathway which further degrades in mineralization. From the whole genome sequencing of *Aspergillus sydowii* found that presence of oxidoreductase, catalytic and hydrolase, activity in bunch of orthologous group has the capability to degrade the PAHs into metabolites of tricarboxylic acid cycle. They degrade the PAHs by the phthalate, catechol and gentistate pathway (Ganesh Kumar *et al.,* 2021). The mitochondrial and cytosolic metabolic pathway of fungi helps to bio transform the xenobiotic PAHs into simpler form. *Aspergillus sydowii* a halophilic fungus has ability to degrade phenanthrene and benzo(a)pyrene from hypersaline medium by forming a lignin modifying enzyme (Peidro-Guzmán *et al.,* 2021). By means of CRISPR/Cas9 technology easily identify these key regulatory networks by disrupting genes and observing resulting phenotypes. By combining this technology with single cell RNA sequencing can get a deep knowledge on how changes of genes affect the cellular processes. Moreover, integration of metabolic modelling with CRISPR-based engineering help to predict the impact of genetic modifications on cellular metabolism, it also enhances the production of specific compounds. Now a days genome-scale and flux balance models

are integrating with CRISPR technology to recognize targets for refining biosynthetic manufacture of metabolites. When suitable targets are recognized, guide RNA forecast replicas used to increase efficiency in gene targeting (Cardiff *et al.,* 2024).

CHALLENGES AND LIMITATIONS

Although integration of CRISPR/Cas9 in mycoremediation has many positive impacts but they also face some challenges in off-target effects, efficiency and precision in no-model fungi and DNA repair mechanism in the target fungi (Nødvig *et al.,* 2015; Schuster & Kahmann, 2019; Wang & Coleman, 2019). Sometimes integration of CRISPR/Cas9 system in fungi might unintentionally edit unintended genomic locations leading to unforeseen consequences. For instance, high level of Cas9 protein causes toxicity in host cell. If the concentration of sgRNA and Cas9 protein are highly expressed then reduction in cell viability can be seen in the organism. In filamentous fungi Cas9 form undesirable double strand breaks and off-target cleavage at unintentional chromosomal loci in high nucleotide identification to the sgRNA sequence (Wang & Coleman, 2019). In some fungi transfer of CRISPR/cas9 components to targeted gene is difficult by using the traditional method. Many CRISPR/Cas9 applications depends on homologous recombination, where the edited DNA is repaired using a homologous DNA template. But sometimes homologous recombination is not as efficient as other DNA repair pathways, which can limit the effectiveness of CRISPR/Cas9 for precise gene editing. Ploidy and multiple copies of genes in fungi are also causes challenges as they all need to be targeted (Zhao *et al.,* 2020).

Biological and ecological limitations are also topic of concerns as there has a possibility that engineered genetic material could move to other microbes in environment and their interactions with other pollutants, soil microbiome and plants is not clearly known. Ethical, legal and biosafety concerns are also causing challenges as releasing of genetically modified fungi into open environments raises ecological and human health concerns such as allergic to other organism and unintended biodegradation of useful materials. Also, many countries have very strict regulations on commercialization of genetically modified organism which limited the field employment and negative views on genetically modified organisms may hinder acceptance of CRISPR/Cas9 based mycoremediation (Ayanoğlu *et al.,* 2020; Kolanu, 2024). Even with all these challenges CRISPR/Cas9 holds great promise in fungal degradation of PAHs.

CONCLUSION AND FUTURE PROSPECTIVE

Over the past decades, CRISPR/Cas9 technology has developed as hopeful edge in the mycoremediation of PAHs. This revolutionary gene editing tool offers extraordinary precision and potentiality for degradation of PAHs by modifying the targeted genes in fungi. This technology has enormous potential for gene knock-out and knock-in to get the desired results for degradation of PAHs. Integration of this promising technology in mycoremediation results a mechanistic framework for upregulation of desired genes for enzyme secretion which has the ability to degrade resistant PAHs. This technology precisely targets and modifies the desired genes to stimulate the production of PAHs degrading enzymes (laccase, peroxidase, cytochrome p450 etc.) by fungi. Overall, CRISPR/Cas9 technology gives a influential tool for revolutionizing mycoremediation by improving fungal capabilities and expanding its application.

But we need to focus more in future research such as multiplex genome editing, enabling simultaneous modification of multiple enzymes system to create synergistic degradation pathways for complex pollutants. For real field application integrating CRISPR-edited fungi into large-scale bioreactors, biofilters, or soil remediation systems could transform current remediation practices into more sustainable, regulatory frameworks, and ecological risk assessments to ensure that engineered strains are safe for environmental release. However, in future research it must be kept in mind that biosafety concerns, regulatory frameworks and ecological risk assessments to ensure that engineered strains are safe for the release in environment.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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