Review Paper: Using genetically modified **Bacillus subtilis BMT4i (MTCC 9447) to Degrade PM2.5 particles.**

Abstract

Interest in the prokaryotic biotransformation of high-molecular-weight polycyclic aromatic hydrocarbons (HMW PAHs) has grown significantly in recent years. Research over the past decade has identified diverse bacterial isolates from various environments with unique metabolic capabilities for HMW PAH biodegradation. However, many of these studies have involved bacteria with pathogenic traits, raising concerns about their practical applications.

Among these bacteria, **Bacillus subtilis** has emerged as one of the most effective bacteria for degrading PAHs, particularly benzo[a]pyrene (BaP). Extensive research has refined the understanding of its degradation pathways, highlighting the role of specific enzymes and metabolic mechanisms.

Bacillus subtilis not only reinforces its effectiveness in degrading harmful pollutants like BaP but also emphasizes its safety and viability for environmental applications, offering a promising solution for mitigating PAH contamination.

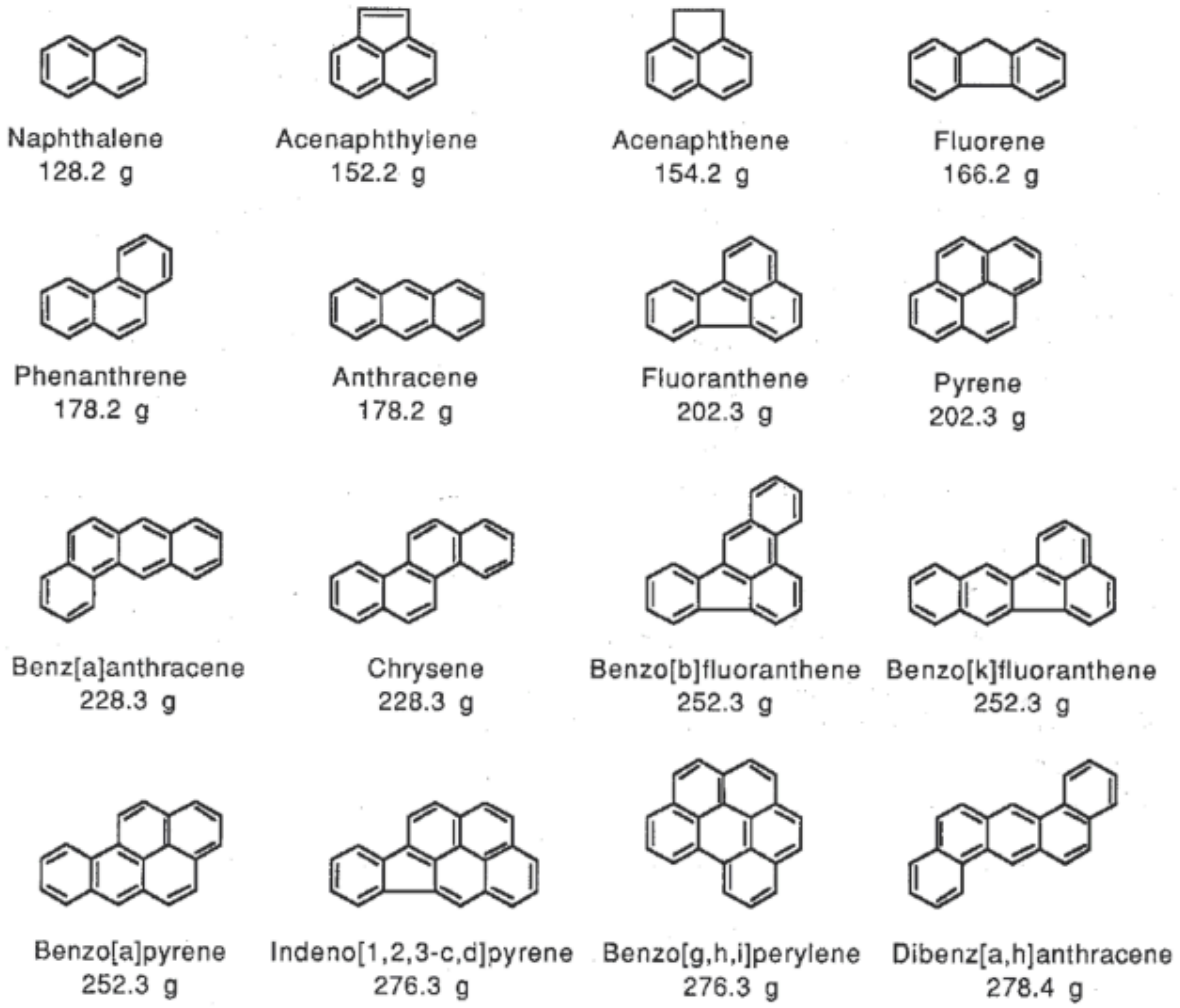
The goal of this review is to provide an outline of the current knowledge of Bacillus subtilis as a potent bioremediation agent, emphasizing its enzymatic pathways, metabolic mechanisms, and the potential for genetic optimization to enhance its efficacy. By exploring its advantages over other bacteria and addressing the need for further research, this review aims to inform future studies and foster the development of safer, more effective strategies for combating PAH contamination in various environments.

Outline of Paper

1. Bacillus Subtilis and its role in degrading PaH

Bacillus subtilis, a Gram-positive bacterium known for its non-pathogenic nature and beneficial applications in agriculture and biotechnology, has been extensively studied for its promising use in bioremediation, particularly in degrading high-molecular-weight polycyclic aromatic hydrocarbons (HMW PAHs) like benzo[a]pyrene (BaP). The strain BMT4i (MTCC 9447) has shown remarkable efficiency in utilizing BaP as a sole carbon and energy source, making it a promising and safer candidate for environmental cleanup efforts. Further research into its metabolic pathways and enzymes could enhance its application for combating PAH contamination.

2. The Problem of Polycyclic Aromatic Hydrocarbons (PAHs)



Polycyclic aromatic hydrocarbons (PAHs) are organic compounds composed of multiple fused aromatic rings. They are primarily formed through the incomplete combustion of organic materials such as coal, oil, gas, and biomass, as well as various industrial activities. PAHs range from simple structures like naphthalene, which has two rings, to more complex compounds like anthracene and phenanthrene, which have three or more rings".

PAHs are ubiquitous pollutants, with some compounds classified on the US Environmental Protection Agency's priority pollutant list. Their persistence in the environment poses significant challenges for cleanup and remediation efforts. While lower-molecular-weight (mol-wt) PAHs are more amenable to bioremediation, higher-molecular-weight PAHs are often recalcitrant to microbial degradation, making them particularly difficult to remove from contaminated sites. The rates of PAH biodegradation can vary widely, influenced by factors such as the PAH structure, site-specific physicochemical conditions, and the types of microorganisms present".

Current research on PAHs emphasizes improving their bioavailability to enhance degradation rates at contaminated sites. However, the byproducts formed during PAH breakdown are not always less toxic than the original compounds. In some cases, these byproducts may still pose environmental risks. To address this, toxicity assays are necessary in bioremediation procedures to assess the safety of

degradation products and ensure remediation efforts effectively remove PAHs while preventing the buildup of harmful substances".

2.1 Environmental and Health Impacts

PAHs are highly toxic, posing significant risks to ecosystems and human health. They exhibit mutagenic and carcinogenic properties, with compounds like benzo[a]pyrene recognized as potent carcinogens. Exposure to PAHs can occur through inhalation of polluted air (e.g., vehicle exhaust or industrial emissions), ingestion of contaminated food, and dermal contact with polluted soil. Chronic exposure has been linked to severe health issues, including respiratory diseases, skin conditions, and an increased risk of cancer".

The persistence and toxicity of PAHs exacerbate their environmental impact. These compounds accumulate in ecosystems, where their degradation is slow, prolonging their harmful effects on biodiversity and public health.

2.2 Challenges in Solving the PAH Problem

Despite numerous studies on PAH degradation, particularly through bioremediation, their persistence remains a significant challenge. Several factors contribute to this:

1. **Chemical Stability and Bioavailability**
   PAHs' hydrophobic nature limits their availability to microorganisms. Bound tightly to soil and sediment particles, they become less accessible for degradation, requiring strategies to enhance their bioavailability".
2. **Microbial and Metabolic Constraints**
   The degradation of PAHs by microorganisms depends on specific metabolic pathways, such as those involving dioxygenases under aerobic conditions. However, these pathways often require optimal environmental conditions, including sufficient oxygen, appropriate pH, and nutrient availability, which are rarely consistent in natural settings".
3. **Genetic Adaptation and Enzymatic Specificity**
   Microorganisms need specialized enzymes to break down high-molecular-weight (HMW) PAHs. While genetic adaptation, such as horizontal gene transfer, enables microbial populations to acquire the necessary catabolic genes, this process is slow and may not always result in efficient degradation. Moreover, the enzymatic systems for degrading HMW PAHs are not well understood, further limiting the effectiveness of bioremediation efforts".
4. **Structural Complexity**
   HMW PAHs are particularly recalcitrant due to their complex molecular structures. Their degradation is not only slower but also more challenging, as fewer microorganisms possess the capability to metabolize these compounds.

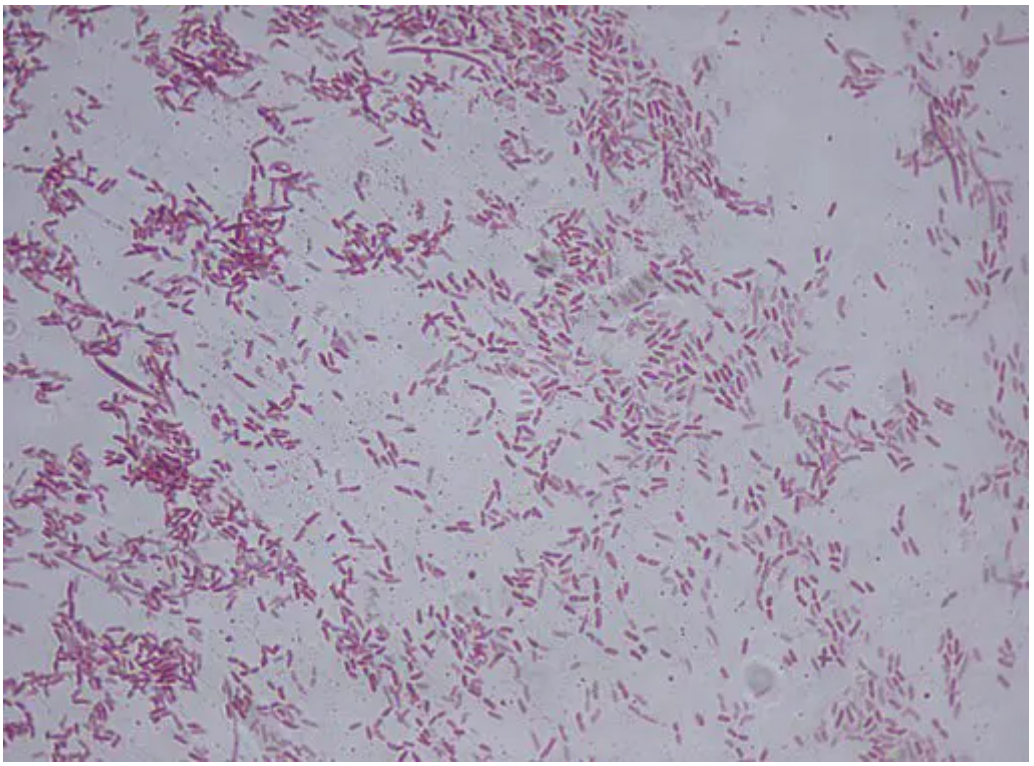
3. Microorganisms for Effective PAH Degradation

3.1 Bacillus subtilis

Bacillus subtilis is widely regarded as the safest microorganism for polycyclic aromatic hydrocarbon (PAH) degradation. This bacterium is considered safe and non-pathogenic, unlike certain other PAH-degrading bacteria such as *Pseudomonas aeruginosa*, which can pose health risks. Furthermore, *B. subtilis* has demonstrated the ability to degrade multiple PAHs simultaneously, including high molecular weight compounds such as pyrene and benzo[a]pyrene. The genetic modification potential of *B. subtilis* is another critical advantage, as it can be engineered to enhance its PAH degradation capabilities while retaining its favorable safety profile. Additionally, studies have shown that *B. subtilis* and *Bacillus sphaericus* can simultaneously degrade both pyrene and benzo[a]pyrene, underscoring their broad degradation abilities and versatility in addressing PAH contamination.

It's important to note that the effectiveness of PAH degradation depends on various factors, including environmental conditions and the specific PAHs involved. While B. subtilis shows promise, it's not universally established as the best or safest option for all PAH bioremediation situations.

3.2 Pseudomonas Species



Pseudomonas species are highly effective in degrading complex PAHs due to their remarkable metabolic versatility and adaptability to various environmental conditions. These bacteria carry genes for PAH degradation on large conjugative plasmids, which facilitates horizontal gene transfer and rapid adaptation to new environmental challenges. Their enzymatic diversity is particularly noteworthy, as they produce a wide range of enzymes, including dioxygenases, that initiate PAH degradation by introducing oxygen into aromatic rings. Moreover, *Pseudomonas* species are capable of utilizing diverse PAHs as carbon and energy sources, including naphthalene, phenanthrene, and even more complex compounds like pyrene. The environmental resilience of these bacteria, which

allows them to thrive in contaminated soils and water bodies, makes them an attractive option for bioremediation applications. However, some *Pseudomonas* species, such as *Pseudomonas aeruginosa*, pose safety concerns due to their opportunistic pathogenicity, which can cause infections in immunocompromised individuals, potentially limiting their use in certain contexts. Additionally, the potential for antibiotic resistance development in some *Pseudomonas* strains raises environmental and public health concerns.

3.3 Escherichia coli

While *Escherichia coli* is genetically versatile and grows rapidly, it is generally less suitable for PAH bioremediation when compared to other microorganisms. The primary limitation lies in its lack of specialized enzymatic pathways required for effective PAH degradation. Unlike *Pseudomonas* species, which possess diverse enzymes capable of breaking down complex aromatic compounds, *E. coli*'s natural metabolic capabilities for PAH degradation are minimal. Furthermore, although *E. coli* can survive in various environments, it faces significant challenges in the harsh conditions often associated with PAH-contaminated sites. As a bacterium primarily adapted to the mammalian gut, it is poorly equipped to thrive in soil or water environments with extreme pH levels, temperature fluctuations, or chemical stressors. While *E. coli* exhibits some catabolic flexibility, it is not as adept as other bacteria in utilizing environmental pollutants as carbon and energy sources. Additionally, although *E. coli* produces RpoS to adapt to stress conditions, its overall stress response is less robust than that of bacteria naturally found in polluted environments, further limiting its suitability for PAH bioremediation.

Nevertheless, while E. coli may not be the most suitable organism for PAH bioremediation research has shown that it can be genetically modified to enhance its PAH degradation capabilities. For example, when specific genes for PAH degradation were introduced into E. coli, its ability to degrade phenanthrene was significantly improved2.

3.4 Yeasts

Yeasts, including *Saccharomyces cerevisiae*, are safe and genetically modified organisms; however, Yeasts generally exhibit limited natural degradation abilities, making them less effective at degrading PAHs. While some strains can utilize various PAHs as carbon and energy sources, their efficiency is lower than specialized bacteria such as *Pseudomonas* species, as they possess fewer specialized enzymes for PAH degradation. Yeasts are considered low-risk organisms due to their low pathogenicity, with many species, especially *S. cerevisiae*, classified as food-grade and safe for human use, which reduces health concerns in bioremediation applications. They adapt to a range of environments but may struggle in harsh, PAH-contaminated sites. Their oxygen requirements are also favorable, using up to 60% less oxygen than activated sludge processes, which can lower operational costs. Furthermore, excess yeast biomass can be repurposed for other applications, and certain yeast strains show promise in heavy metal remediation, making them useful in environments with multiple pollutants.

3.5 Bacillus cereus

Bacillus cereus produces enzymes, such as cellulase, that are capable of degrading PAHs, including complex organic compounds. However, its effectiveness in PAH degradation varies significantly between strains due to differences in biofilm formation and enzymatic activity. While sporulation allows *B. cereus* to survive in harsh conditions, this trait can also make the bacteria more resistant to removal, potentially leading to persistent contamination. Despite its potential in bioremediation, the safety concerns surrounding *B. cereus* cannot be overlooked. As a known pathogen, *B. cereus* poses significant health risks, including the production of toxins that could impact the environment and human health. These factors, combined with inconsistent degradation capabilities between strains, complicate its use in bioremediation applications, particularly in open environments where its pathogenicity and toxin production present challenges.

The reason I chose to focus my research primarily on Bacillus subtilis for bioremediation lies in its unique combination of advantages. While it hasn't been universally declared the best option for PAH degradation, B. subtilis offers a promising balance of effectiveness and safety. It has inherent capabilities to degrade PAH particles, including high molecular weight compounds, and can do so without the pathogenic risks associated with other microorganisms like Bacillus cereus or Pseudomonas aeruginosa. Moreover, its genetic modifiability makes it adaptable for enhanced degradation performance.

4. Distinctive Attributes of Bacillus subtilis BMT4i in BaP Degradation

Bacillus subtilis BMT4i showcases unique capabilities in the degradation of benzo(a)pyrene (BaP), with a pathway that is chromosomally encoded, meaning the genes responsible for this function are integrated into the bacterium's chromosome rather than carried on plasmids. This was confirmed through experiments demonstrating that removing plasmids had no impact on BaP degradation and which I will talk about later. The degradation pathway is inducible, which implies it can be activated under specific environmental conditions; for example, chloramphenicol induction significantly enhanced the rate of degradation, suggesting that certain factors can boost its biodegradation efficacy. Optimal conditions for the highest degradation rates include a temperature of 30°C, a pH of 8.0, and the presence of surfactants like Tween-20, which promote robust bacterial growth and metabolic processes. During the degradation, various metabolites, including benzo(a)pyrene-11,12-epoxide and 8-carboxy-7-hydroxy pyrene, are produced, pointing to the involvement of multiple enzymatic pathways such as monooxygenases and dioxygenases in breaking down BaP 3.

Moving forward, I will discuss experimental studies that provide further insights into the effectiveness and potential of B. subtilis BMT4i as a bioremediation agent.

5. Studies done on Bacillus Subtilis

5.1 Degradation of Benzo [a] Pyrene by a novel strain *Bacillus subtilis* BMT4i (MTCC 9447)

The study aimed to isolate bacteria capable of utilizing benzo[a]pyrene (BaP) as a sole source of carbon and energy, resulting in the identification of a novel strain, *Bacillus subtilis* BMT4i (MTCC 9447), from automobile-contaminated soil. Using enrichment culture techniques, researchers cultivated bacterial colonies in a basal salt mineral (BSM) medium containing BaP (50 µg/mL). Among the isolates, BMT4i emerged as the most efficient degrader, with growth kinetics monitored through the colony-forming unit (CFU) method, revealing a 1029-fold increase in biomass within seven days. High-performance liquid chromatography (HPLC) was used to analyze BaP degradation products, showing that BMT4i degraded approximately 84.66% of BaP over 28 days.

Interestingly, BMT4i demonstrated extended viability in the BSM medium for up to 40 days, a trait not previously reported in other bacteria. Unlike prior studies, where BaP degradation occurred only co-metabolically in the presence of additional carbon sources, BMT4i efficiently utilized BaP as its sole carbon source. HPLC analysis also detected six intermediate metabolites after seven days of incubation, although their identities are still under investigation.

The study highlighted the adaptability of BMT4i, which can also degrade other polycyclic aromatic hydrocarbons (PAHs) such as naphthalene, anthracene, and dibenzothiophene.

These findings highlight the suitability of BMT4i as a robust candidate for bioremediation, particularly in environments contaminated with PAHs. Given its origin in automobile-contaminated soil and ability to thrive in BaP-rich conditions, BMT4i could serve as a model organism for developing strategies to mitigate PAH pollution and reduce environmental contamination effectively.

5.2 Optimization of an inducible, chromosomally encoded benzo [a] pyrene (BaP) degradation pathway in *Bacillus subtilis* BMT4i (MTCC 9447)

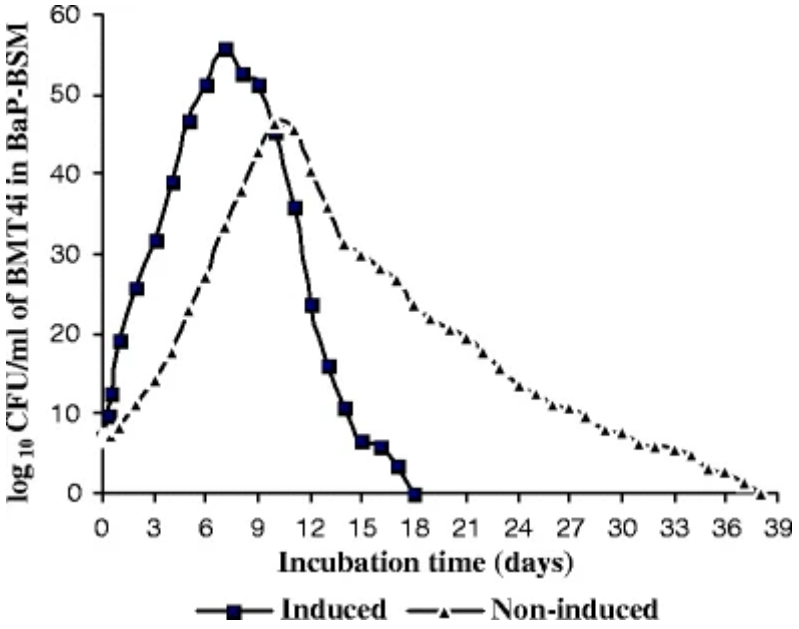
In this study researchers isolated a novel strain, *Bacillus subtilis* BMT4i (MTCC 9447), capable of utilizing BaP (benzo[a]pyrene) as the sole carbon and energy source. They investigated whether the bacterium's ability to degrade BaP could be induced and determined if the genes responsible for this degradation were located on plasmids or the bacterial chromosome. A series of experiments assessed the impact of various physical and chemical factors on BaP degradation, including temperature, pH, UV light exposure, BaP concentration, surfactants, and ionic strength. It was observed that the bacterium degraded BaP ten times faster in induced cultures compared to non-induced ones, confirming that the BaP degradation pathway is inducible and can be activated or enhanced by external stimuli.

The absence of plasmids and the maintenance of BaP degradation ability after plasmid curing led to the conclusion that the genes encoding the BaP degradation pathway are located on the bacterium's chromosomal DNA. This chromosomal localization is advantageous for bioremediation, as chromosomal traits are stable, unlikely to be lost during bacterial replication, and reduce the risk of horizontal gene transfer to other microorganisms in the environment.

Optimal conditions for BaP degradation by *B. subtilis* BMT4i were identified, with maximum activity observed at a temperature of 30°C, a pH of 8.0, and the presence of 0.01% Tween-20 as a surfactant. Magnesium sulfate concentrations between 400–1,800 µM further enhanced degradation efficiency.

The degradation process also benefited from UV-induced photolysis, which facilitated the breakdown of BaP.

To further characterize this degradation capability, researchers conducted experiments comparing induced and non-induced cultures. After induction, cells were washed and inoculated into minimal medium supplemented with BaP and chloramphenicol to inhibit protein synthesis. Cultures were grown at 37°C, and bacterial growth and BaP degradation were monitored using colony-forming unit (CFU) counts and high-performance liquid chromatography (HPLC). Induced cultures demonstrated significantly enhanced BaP degradation rates compared to non-induced ones, highlighting the inducibility of the degradation pathway.



The data confirms the inducible nature of the BaP degradation pathway in *B. subtilis* BMT4i. HPLC analysis showed that induced cultures achieved over 90% BaP degradation, compared to 84% in non-induced cultures

The long-term degradation study revealed that induced cultures achieved over 80% BaP degradation after 28 days, coupled with a 1,029-fold increase in cell number within seven days. Metabolic analysis identified degradation intermediates, including benzo[a]pyrene-11,12-epoxide and 8-carboxy-7-hydroxy pyrene, indicating the involvement of multiple enzymatic pathways, such as monooxygenases and dioxygenases, in BaP breakdown.

These findings establish *B. subtilis* BMT4i as a promising candidate for bioremediation strategies targeting polycyclic aromatic hydrocarbon (PAH)-contaminated environments. Its stable chromosomal trait for BaP degradation, coupled with its ability to degrade BaP under optimized conditions, underscores its potential for effective cleanup of polluted sites.

5.3 Benzo(a)pyrene degradation pathway in *Bacillus subtilis* BMT4i (MTCC 9447)

Benzo(a)pyrene (BaP) is a high-molecular-weight polycyclic aromatic hydrocarbon (PAH) known for its persistence and toxic effects in the environment. Bacillus subtilis BMT4i (MTCC 9447) has shown potential for degrading BaP, but the exact mechanisms involved remain underexplored. This study aimed to determine the degradation pathway through a series of experiments that utilized

ultra-high-performance liquid chromatography-mass spectrometry (UHPLC-MS) for metabolite identification.

The bacterial strain was cultured for 15 days in a nutrient-rich medium containing BaP. Samples were periodically collected and analyzed by UHPLC-MS to track the formation and identification of metabolic intermediates. The analysis identified a range of compounds, starting with benzo(a)pyrene-11,12-epoxide as a key early product, generated by cytochrome P450 monooxygenase activity. This intermediate underwent further transformation into benzo(a)pyrene-cis-7,8-dihydrodiol and 7,8,9,10-tetrahydrobenzo[pqr]tetraphene-7,8,9,10-tetraol, suggesting oxidation at C-11, C-12, C-7, C-8, C-9, and C-10 positions by enzymes such as monooxygenases and dioxygenases.

Subsequent stages of the degradation pathway revealed the presence of more complex, ring-cleaving metabolites. Compounds such as 8-carboxy-7-hydroxy pyrene and chrysene-4 or 5-carboxylic acid indicated the oxidative cleavage of BaP's aromatic rings. Additionally, the metabolite cis-4-(8-hydroxypyrene-7yl)-2-oxobut-3-enoic acid was identified, supporting the notion of advanced degradation beyond simple oxidation. Traces of residual BaP and partially transformed products like hydroxymethyl benzo(a)pyrene were also observed, suggesting that complete mineralization was not achieved in the given conditions.

The results confirmed that B. subtilis BMT4i can break down BaP through an enzymatic pathway involving oxidative and ring-cleaving reactions. These findings are significant for bioremediation strategies targeting PAH-contaminated environments. The discovery of specific metabolic intermediates and the enzymes likely involved, such as cytochrome P450 monooxygenases, provide insight into the mechanisms that could be leveraged for enhanced degradation. However, the incomplete mineralization of BaP indicates that further metabolic steps might be necessary to achieve full detoxification.

In summary, B. subtilis BMT4i demonstrates a significant capacity for degrading BaP through a well-defined pathway that includes oxidation, ring cleavage, and formation of less harmful intermediates. This work highlights the potential of using B. subtilis as a bioremediation agent and points to the need for further research to achieve complete mineralization and assess the full ecological impact of the degradation products.

5.4 Identification and characterization of novel bacterial polyaromatic hydrocarbon-degrading enzymes as potential tools for cleaning up hydrocarbon pollutants from different environmental sources

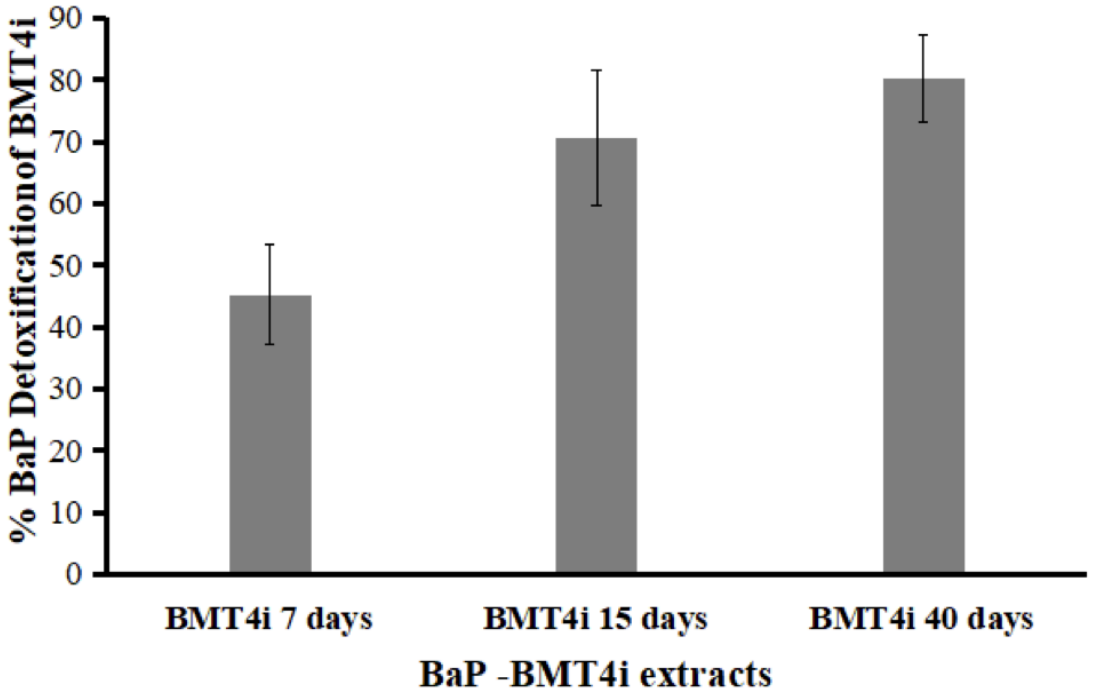
In this study, four bacterial strains were isolated from contaminated soil using mineral salt medium (MSM) with anthracene, alpha-naphthalene, or catechol as the sole carbon sources. These strains were identified through 16S rRNA sequencing as *Bacillus anthracis*, *B. cereus*, *B. mojavensis*, and *B. subtilis*. The degradation capacities of these isolates were assessed using HPLC analysis. Among them, *B. subtilis* demonstrated the highest degradation efficiency for anthracene, reaching 99% after five days of incubation.

Additionally, *B. subtilis* exhibited the highest activity of catechol 1,2-dioxygenase when cultured in MSM with anthracene. The enzyme was purified by gel filtration chromatography and characterized, showing a molecular weight of 70 kDa, a Km value of 2.7 µg, and a Vmax of 178 U/mg protein. The gene encoding catechol 1,2-dioxygenase was isolated from all four strains and submitted to GenBank

(accession numbers MG255165-MG255168). During a 72-hour incubation period, the expression of this gene in *B. subtilis* was upregulated by a factor of 23.2.

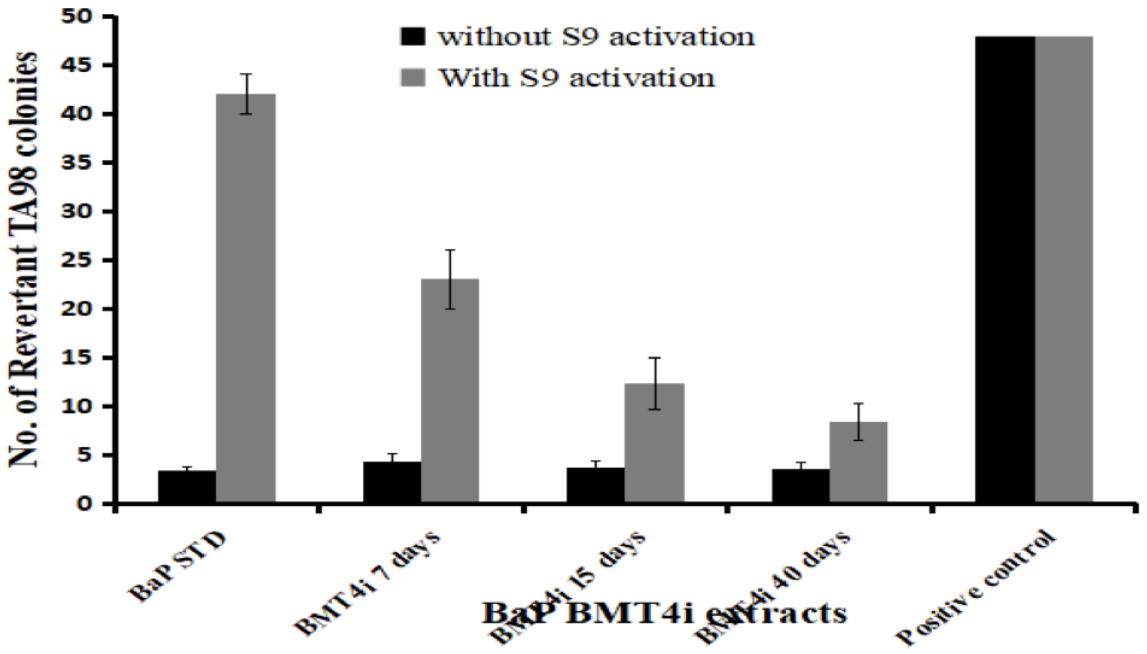
5.5 Benzo (A) Pyrene Detoxification Potential of Bacillus subtilis BMT4i (MTCC 9447) Isolated From Srinagar, Garhwal, Uttarakhand, India

In the study *"Benzo(a)Pyrene Detoxification Potential of Bacillus subtilis BMT4i (MTCC 9447) Isolated From Srinagar, Garhwal, Uttarakhand, India,"* Bacillus subtilis BMT4i demonstrated significant potential in detoxifying benzo(a)pyrene (BaP), a hazardous environmental contaminant with carcinogenic properties. The key findings highlighted BMT4i's efficiency in degrading BaP, showing reductions in BaP concentration of 68%, 78%, and 89% after 7, 15, and 40 days of incubation, respectively, compared to negative controls.



% BaP detoxification after 7, 15 and40 days of BaP-BMT4i incubation

This aligns with previous research supporting the bacterium's ability to break down BaP. Mutagenicity assessments using the Ames test with the TA98 strain of *Salmonella typhimurium* showed that the BaP-BMT4i extracts did not exhibit mutagenic effects in the absence of S9 activation, indicating no frameshift mutations. However, with S9 activation, the mutagenicity of the BaP standard was significantly higher than in the test extracts, with revertant colonies decreasing from 43/48 in the BaP standard to 23/48, 12.4/48, and 8.3/48 in the 7, 15, and 40-day extracts, respectively, demonstrating BMT4i's potential in significantly reducing BaP mutagenicity.



BaP-BMT4i extracts induced revertant TA98 colonies with or without S9 activation

The detoxification potential of BMT4i was quantified, showing 45% detoxification after 7 days, increasing to 70% after 15 days and 80% after 40 days, which underscored its progressive efficiency over time. Nevertheless, some mutagenicity remained in the BMT4i-BaP extracts, possibly due to residual BaP or the formation of intermediate, potentially mutagenic metabolites, whose identification is difficult due to the lack of commercial standards and known absorbance characteristics.

The in-vitro detoxification results indicate that BMT4i holds promise as a bioremediation agent for BaP-contaminated sites, but further field trials are needed to verify its effectiveness and safety in practical applications. This suggests that Bacillus subtilis BMT4i could be an effective tool for the bioremediation of environments contaminated with high molecular weight polyaromatic hydrocarbons like BaP.

6. Enzymatic Pathways and Mechanisms of Polycyclic Aromatic Hydrocarbon (PAH) Degradation by *Bacillus subtilis*

Bacillus subtilis produces several enzymes that contribute to the degradation of PAHs, though the precise pathways and mechanisms are not fully characterized. The key enzymes involved in PAH degradation likely include dioxygenases or monooxygenases, which are responsible for the initial oxidation of PAHs by adding hydroxyl groups to the aromatic rings and facilitating further reactions. Dehydrogenases play an essential role in converting dihydrodiol intermediates into diol compounds, allowing the ring structure to rearomatize. Ring-cleaving dioxygenases are another critical group of enzymes that cleave the aromatic rings of PAHs, which can occur through either ortho-cleavage or meta-cleavage pathways. Additionally, catechol-degrading enzymes are involved in breaking down the catechol intermediates produced after the ring cleavage.

Bacillus subtilis has been shown to possess the potential for degrading polycyclic aromatic hydrocarbons (PAHs) through a series of enzymatic reactions. While the specific pathways remain incompletely characterized, it is known that *B. subtilis* likely utilizes enzymes that are also found in other PAH-degrading organisms, such as lignin peroxidase and P450 monooxygenases. However, comprehensive research is still needed to fully map out these pathways in *B. subtilis*.

The degradation of PAHs typically starts with the initial oxidation of the aromatic molecule, a process likely carried out by dioxygenases or monooxygenases. These enzymes introduce hydroxyl groups into the PAH structure, setting the stage for further breakdown. This initial oxidation step leads to the formation of a cis-dihydrodiol intermediate, which is a product that contains two additional hydroxyl groups added to the ring. The next step involves rearomatization, where dehydrogenase enzymes convert this intermediate into a stable diol compound, restoring the aromatic structure.

Following this, ring-cleaving dioxygenases play a crucial role by breaking the aromatic ring open through either intradiol (ortho-cleavage) or extradiol (meta-cleavage) pathways. This ring cleavage produces catechol intermediates, which are then further broken down by catechol-degrading enzymes. The breakdown of catechols leads to the formation of tricarboxylic acid (TCA) cycle intermediates, which the bacteria can use as energy sources. This step supports *B. subtilis*'s metabolic processes and growth, demonstrating its ability to utilize PAHs as carbon sources.

Research has shown that *B. subtilis* has the ability to transform PAHs such as pyrene and benzo[a]pyrene, although the specific enzymes and full metabolic pathways remain under investigation. In one study, *B. subtilis* demonstrated the ability to transform approximately 40% of pyrene and 50% of benzo[a]pyrene over four days''. This suggests that *B. subtilis* may share similarities with other PAH-degrading bacteria that utilize enzymes like lignin peroxidase and P450 monooxygenases. However, further research is needed to fully understand the specific enzymatic mechanisms and pathways that *B. subtilis* employs in PAH degradation.

7. Future Directions for Bioremediation

The next steps for bioremediation using *Bacillus subtilis* for PAH degradation should include assessing the toxicity of metabolites produced during PAH transformation to ensure safety, conducting mineralization studies to determine if *B. subtilis* can fully convert pyrene and benzo[a]pyrene to carbon dioxide and water, and enhancing PAH degradation capabilities through genetic optimization by improving key enzyme expression.

Additionally, field trials in contaminated sites are necessary to test effectiveness under real-world conditions. Exploring mixed microbial systems (MMS) by combining *B. subtilis* with other safe microorganisms could increase efficiency, while improving PAH bioavailability through biosurfactants or other agents should be investigated. Further elucidation of the metabolic pathways and enzymes involved in PAH degradation and assessing long-term stability in contaminated environments will help develop *B. subtilis* as a safe, effective bioremediation tool.

Current research highlights the need for genetic optimization of *Bacillus subtilis* as studies largely focus on its natural degradation capabilities. Genetic engineering holds promise for enhancing the bacterium's ability to break down PAHs and other pollutants. By introducing or upregulating specific genes linked to PAH degradation, researchers can develop strains with improved metabolic efficiency for processing these harmful compounds. Additionally, targeted modifications of metabolic pathways,

such as adjusting enzyme production or activity, can facilitate the breakdown of complex PAH structures that are otherwise challenging to degrade.

Genetic enhancements can also make *B. subtilis*'s tolerance to toxic pollutants, which is vital for effective bioremediation in environments with high concentrations of PAHs that may otherwise hinder microbial activity. Moreover, modifications can increase the adaptability of the strain to harsh conditions, including extreme pH levels or temperatures commonly present in contaminated sites, ensuring survival and functionality in various habitats. Studies indicate that engineered strains exhibit higher rates of PAH transformation compared to their wild-type counterparts, with the potential to utilize a broader range of PAHs as carbon sources, thus enhancing the overall efficiency of bioremediation efforts. The development of artificial mixed microbial systems (MMS) with optimized strains could further offer an innovative approach to improving PAH degradation outcomes.

8. Point of view

From my perspective, advancing our understanding of the enzymes and metabolic pathways responsible for PAH degradation is essential. While continuing this research, scientists can also build upon their current knowledge by exploring ways to optimize what has already been discovered. One approach could be the overexpression of cytochrome P450 monooxygenases, a family of enzymes that *B. subtilis* naturally encodes. According to research, *B. subtilis* has eight cytochrome P450 enzymes: BioI (CYP107H1), CypA (CYP107J1), CypC (CYP152A1), CypX (CYP134A1), PksS (CYP107K1), YetO (CYP102A2), YjiB (CYP109B1), and YrhJ (CYP102A3). These enzymes play a significant role in various oxidation reactions and metabolic processes within the bacterium.

The overexpression of certain enzymes in bacteria can significantly enhance bioremediation efficiency for several reasons. First, increased enzyme availability leads to higher concentrations of specific enzymes within the bacterial cells. This allows for more rapid and efficient breakdown of pollutants such as PAHs, enabling the processing of a greater number of substrate molecules simultaneously.

In addition, enzyme overexpression can lead to enhanced catalytic activity. Even if the inherent activity of the enzymes remains the same, the higher number of active sites available for reactions can result in an increased overall rate of pollutant degradation. This means that bacteria can process PAHs more effectively, contributing to the success of bioremediation efforts.

Moreover, Another key benefit of overexpressing specific enzymes is the potential for increased resistance to toxic compounds. Overexpressing enzymes that help detoxify or process harmful substances can enable bacteria to tolerate higher concentrations of toxic pollutants present in contaminated environments. This resilience allows engineered strains to thrive and continue degrading pollutants, even under harsh conditions.

Overall, overexpressing cytochrome P450 genes in *B. subtilis* can optimize its capacity for PAH degradation, improve bioremediation performance, and enhance its adaptability to contaminated environments. This approach holds promise for creating strains that are better equipped to address environmental pollution and contribute to more effective bioremediation strategies.

9. Conclusion

Bacillus subtilis stands out as a promising candidate for bioremediation, particularly due to its natural ability to break down PAHs, which are persistent and toxic pollutants posing serious threats to

ecosystems and human health. The five studies we reviewed highlighted the effectiveness of specialized strains like BMT4i in degrading hazardous compounds such as benzo[a]pyrene, showcasing what a powerful tool it is in environmental remediation. However, to harness its full capacity, further detailed investigations into the specific enzymes and metabolic pathways are essential.

To maximize its bioremediation capabilities, genetic optimization is seen as essential. By overexpressing key enzymes, such as cytochrome P450 monooxygenases, the availability and catalytic activity of these enzymes can be significantly increased, leading to more effective breakdown of PAHs. This approach may facilitate more robust responses to toxic pollutants and improve metabolic efficiency, enabling B. subtilis to thrive in diverse and challenging environments.

While B. subtilis has shown significant promise, it is evident that continued research and targeted genetic advancements are necessary to create strains with improved adaptability, higher degradation rates, and greater resistance to environmental stressors. Integrating current understanding with innovative genetic engineering is a necessary step for addressing the growing environmental challenges posed by PAH contamination and safeguarding the health of us and of our planet.

Sources

- Environmental aspects of PAH biodegradation
- Comparative Transcriptomics Reveals Adaptive Changes in Cellular Metabolism Of Bacillus Subtilis MSC4 Under the Stress of Benzo[A]Pyrene
- The study of growth kinetics of Bacillus subtilis BMT4i (MTCC 9447) using diesel as the sole carbon and energy source
- Microbial biodegradation of polyaromatic hydrocarbons
- Bacterial Degradation and Bioremediation of Polycyclic Aromatic Hydrocarbons
- Current State of Knowledge in Microbial Degradation of Polycyclic Aromatic Hydrocarbons (PAHs): A Review
- Bacterial Degradation of Aromatic Compounds
- Efficient bioremediation of PAHs-contaminated soils by a methylotrophic enrichment culture
- Evidence for novel polycyclic aromatic hydrocarbon degradation pathways in culturable marine isolates
- Pseudomonas veronii strain 7–41 degrading medium-chain n-alkanes and polycyclic aromatic hydrocarbons
- Artificial mixed microbial system for polycyclic aromatic hydrocarbons degradation
- Biodegradation of Benzo[a]pyrene by a White-Rot Fungus Phlebia acerina: Surfactant-Enhanced Degradation and Possible Genes Involved
- Degradation of Benzo [a] Pyrene by a novel strain *Bacillus subtilis* BMT4i (MTCC 9447)
- Optimization of an inducible, chromosomally encoded benzo [a] pyrene (BaP) degradation pathway in *Bacillus subtilis* BMT4i (MTCC 9447)
- Benzo(a)pyrene degradation pathway in *Bacillus subtilis* BMT4i (MTCC 9447)
- Identification and characterization of novel bacterial polyaromatic hydrocarbon-degrading enzymes as potential tools for cleaning up hydrocarbon pollutants from different environmental sources
- Benzo (A) Pyrene Detoxification Potential of Bacillus subtilis BMT4i (MTCC 9447) Isolated From Srinagar, Garhwal, Uttarakhand, India
- Bacillus subtilis is a Potential Degrader of Pyrene and Benzo[a]pyrene
- The crystal structure of the versatile cytochrome P450 enzyme CYP109B1 from Bacillus subtilis