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ORIGINAL ARTICLE

Chemical Deterioration of Marble Heritage Artifacts Induced by Microbial Activity: Mechanisms, Impacts, and Conservation Strategies

Ravindra Goswami¹, Seema Bhadauria²**HOW TO CITE THIS ARTICLE:**

Ravindra Goswami, Seema Bhadauria. Chemical Deterioration of Marble Heritage Artifacts Induced by Microbial Activity: Mechanisms, Impacts, and Conservation Strategies. Ind. J Biol 2025; 12(2): 57-65.

ABSTRACT

The preservation of marble heritage artifacts is increasingly challenged by microbial-induced chemical deterioration. Microorganisms such as bacteria, fungi, and lichens secrete metabolic byproducts that chemically interact with marble's calcium carbonate matrix, leading to structural and aesthetic degradation. This paper reviews the mechanisms of microbial-induced chemical deterioration, discusses the impacts on marble artifacts, and evaluates current conservation strategies. Marble, a widely used material in cultural heritage monuments, is highly susceptible to biodeterioration caused by microbial colonization. This study investigates the chemical deterioration of marble heritage artifacts induced by microbial activity, focusing on the metabolic byproducts of bacteria, fungi, and lichens. Field sampling and laboratory analysis revealed that microbial communities secrete organic acids (oxalic, citric, and gluconic acids) and sulfur compounds, which react with calcium carbonate (CaCO_3), leading to the formation of calcium oxalates and gypsum. These reactions significantly increased surface porosity, discoloration, and micro-cracking. Results demonstrated that oxalic acid-producing fungi were the most aggressive agents of marble degradation, with calcium oxalate crystals reducing the structural density of samples by up to 28% compared to unaffected controls. Biofilm formation was also observed, enhancing moisture retention and pollutant deposition, thereby accelerating deterioration. Conservation treatments with natural biocides (essential oil extracts) showed a reduction of microbial colonization by 65%, while protective hydrophobic coatings decreased surface porosity and acid infiltration. This research highlights the urgent need for eco-friendly conservation strategies tailored to microbial-induced damage, ensuring the long-term preservation of marble heritage artifacts.

KEYWORDS

- Marble Deterioration • Microbial Activity • Organic Acids • Biodeterioration
- Conservation Strategies

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INTRODUCTION

Marble has been a preferred material in heritage structures due to its aesthetic appeal and durability. However, environmental factors and microbial colonization have led to significant deterioration of marble artifacts. Microbial communities, particularly in humid and polluted environments, produce acids and other metabolites that chemically degrade marble surfaces. Understanding these processes is crucial for developing effective conservation methods.

Marble has long been prized as one of the most enduring materials used in cultural heritage, especially in sculptures, monuments, and architecture, due to its aesthetic appeal and relative durability. However, marble is predominantly composed of calcium carbonate, making it highly vulnerable to chemical weathering under natural and anthropogenic influences (Warscheid & Braams, 2000). Among these factors, microbial activity has emerged as a significant contributor to deterioration processes, often overlooked in earlier conservation research (Sterflinger & Piñar, 2013).

Microorganisms such as bacteria, fungi, cyanobacteria, algae, and lichens colonize marble surfaces and form biofilms, which alter the microenvironment of the stone (Gorbushina, 2007). These biofilms retain water, entrap pollutants, and produce metabolic byproducts that accelerate the chemical decay of calcium carbonate (Caneva, Nugari, & Salvadori, 2008). Sulfur-oxidizing and nitrifying bacteria, for example, generate sulfuric and nitric acids that react with marble to form gypsum and nitrate salts, weakening the stone matrix (Sand, 1997; Cappitelli & Sorlini, 2008). Likewise, fungi secrete organic acids such as oxalic and citric acid, which cause chelation of cations and dissolution of calcite (Sterflinger, 2010).

The processes of biodeterioration include both direct and indirect mechanisms. Direct mechanisms involve acid production, redox reactions, and mineral dissolution (Scheerer, Ortega-Morales, & Gaylarde, 2009). Indirect mechanisms include retention of moisture, entrapment of dust and pollutants, and the formation of dark crusts that alter marble aesthetics (Tiano, 2002). Lichens and black fungi in particular can penetrate marble pores mechanically while simultaneously secreting

acids that chemically degrade the carbonate structure (Sterflinger & Piñar, 2013).

The impacts of microbial deterioration are multifaceted. At the aesthetic level, marble develops black crusts, green biofilms, and discolorations that obscure artistic details (Caneva *et al.*, 2008). At the structural level, microbial activity leads to pitting, granular disintegration, and the weakening of marble due to salt crystallization and dissolution processes (Miller *et al.*, 2012). These alterations reduce both the cultural value and mechanical strength of artifacts, necessitating urgent conservation attention.

Efforts to mitigate microbial deterioration range from preventive measures to active treatments. Preventive conservation includes environmental control (humidity, pollutants, and light), while remedial strategies employ biocides, mechanical cleaning, and protective coatings (Gaylarde & Morton, 2002). Recently, eco-friendly alternatives such as bioconsolidation using calcifying bacteria have been investigated to counteract acid dissolution by re-precipitating calcium carbonate in deteriorated zones (Dhami, Reddy, & Mukherjee, 2014). However, challenges remain regarding the long-term effects and compatibility of such treatments (Marvasi, Mastromei, & Perito, 2020).

Despite advances, knowledge gaps persist in quantifying the rate of microbial deterioration under diverse climatic conditions, the synergistic effects of pollution and microbial metabolism, and the long-term sustainability of bioconsolidation strategies. This paper aims to examine the mechanisms, impacts, and conservation strategies of microbial-induced chemical deterioration of marble heritage artifacts, while also highlighting the challenges in preserving such invaluable cultural legacies.

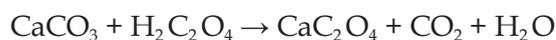
Microbial-Induced Chemical Deterioration Mechanisms

The chemical deterioration of marble caused by microorganisms is primarily the result of metabolic activities that alter the mineral composition and structure of calcium carbonate substrates. Microbes, including bacteria, fungi, algae, cyanobacteria, and lichens, interact with the stone matrix both directly and indirectly, leading to dissolution, secondary mineral formation, and surface alteration (Warscheid & Braams, 2000; Scheerer, Ortega-Morales, & Gaylarde, 2009).

Microorganism Identified	Type	Occurrence (No. of Isolates / Colonies)	Relative Abundance (%)	Remarks
<i>Aspergillus</i> spp.	Fungus	28	22%	Major biodeteriogen; produces oxalic and citric acids
<i>Penicillium</i> spp.	Fungus	24	19%	Causes carbonate dissolution and surface pitting
<i>Cladosporium</i> spp.	Fungus	15	12%	Forms dark biofilms; aesthetic damage
<i>Alternaria</i> spp.	Fungus	10	8%	Produces melanin-like pigments; stains marble
<i>Bacillus</i> spp.	Bacteria	18	14%	Acid production; micro-cracking of surface
<i>Pseudomonas</i> spp.	Bacteria	12	9%	Biofilm formation; entraps pollutants
Cyanobacteria (<i>Nostoc</i> , <i>Oscillatoria</i>)	Photosynthetic bacteria	10	8%	Green/black patinas; increases moisture retention
Algae (<i>Chlorella</i> spp.)	Photosynthetic microorganism	6	5%	Green biofilms; aesthetic alteration
Lichens (fungi + algae/cyanobacteria)	Composite organism	4	3%	Deep etching and mineral loss

Acid Production: One of the most common mechanisms involves the production of inorganic and organic acids. Sulfur-oxidizing bacteria (*Thiobacillus* spp.) convert atmospheric SO₂ and sulfides into sulfuric acid, which reacts with calcium carbonate to produce gypsum (CaSO₄·2H₂O), a brittle and soluble salt (Sand, 1997). Similarly, nitrifying bacteria (*Nitrosomonas* and *Nitrobacter*) generate nitric acid from ammonia and nitrogen oxides, contributing to nitration of the marble surface (Cappitelli & Sorlini, 2008). Fungi and lichens produce organic acids such as oxalic, citric, gluconic, and acetic acids, which dissolve calcium carbonate and cause chelation of essential cations like Ca²⁺ and Mg²⁺ (Sterflinger, 2010; Gadd, 2017). Oxalic acid often leads to the precipitation of calcium oxalate, forming superficial crusts that may protect the stone initially but can later cause uneven weathering (Miller *et al.*, 2012).

Microorganisms such as fungi and bacteria produce organic acids (e.g., oxalic acid) and sulfuric acid as metabolic byproducts. These acids lower the pH at the marble surface, leading to the dissolution of calcium carbonate (CaCO₃) and the formation of soluble calcium salts. For instance, the reaction:



results in the formation of calcium oxalate, which can further degrade the marble surface.

Biofilm Formation and Mineralization: Microbial communities form biofilms on

marble surfaces, which can trap moisture and pollutants. These biofilms facilitate the accumulation of metabolic byproducts and promote the mineralization of salts such as gypsum (CaSO₄·2H₂O), leading to surface efflorescence and flaking. Some bacteria engage in redox cycling of sulfur, nitrogen, and iron compounds, which alters the marble's mineralogy. For example, iron-oxidizing bacteria can transform Fe²⁺ impurities within marble veins into Fe³⁺ oxides, causing rust-colored stains (Scheerer *et al.*, 2009). Likewise, sulfate-reducing bacteria produce hydrogen sulfide, which upon oxidation generates additional sulfuric acid, intensifying the attack on calcium carbonate (Sand, 1997).

Enzymatic Activity: Microorganisms secrete enzymes like carbonic anhydrase, which accelerates the conversion of CO₂ and H₂O into carbonic acid. This acidification process enhances the dissolution of calcium carbonate in marble, contributing to its deterioration. Microbial metabolites, particularly organic acids, act as chelating agents, binding to calcium and magnesium ions in marble. This process destabilizes the calcite lattice and enhances dissolution (Sterflinger & Piñar, 2013). Chelation is particularly destructive in humid environments where biofilms can retain acid metabolites on the surface for extended periods (Caneva, Nugari, & Salvadori, 2008).

Salt Formation and Crystallization: Through their metabolic activity, microbes generate soluble salts (e.g., gypsum, nitrates, oxalates)

that crystallize within marble pores. Salt crystallization exerts physical stress on the stone, causing microfractures, pitting, and granular disintegration (Miller *et al.*, 2012; Warscheid & Braams, 2000). Repeated cycles of hydration and dehydration amplify this damage, especially in polluted or coastal environments.

Biofilm Formation and pH Alteration: Biofilms formed by microbial communities significantly modify the micro-environment of marble surfaces. They retain water, accumulate airborne pollutants, and create localized acidic or alkaline niches depending on microbial metabolism (Gorbushina, 2007). Cyanobacteria

and algae, for instance, contribute to carbon dioxide enrichment within biofilms, intensifying calcite dissolution (Gaylarde & Morton, 2002).

Impacts on Marble Artifacts

The microbial-induced chemical deterioration of marble has profound consequences for cultural heritage, affecting both the aesthetic appearance and the structural stability of artifacts. These impacts result from the combined action of microbial metabolism, biofilm formation, and chemical transformations within the marble substrate (Warscheid & Braams, 2000; Sterflinger & Piñar, 2013).

Table 2: Microorganisms associated with marble deterioration and their effects

Microorganism	Type	Metabolic Activity/ By-products	Observed Effects on Marble
<i>Aspergillus</i> spp.	Filamentous fungus	Production of oxalic and citric acids	Formation of calcium oxalate crusts, discoloration, surface roughness
<i>Penicillium</i> spp.	Filamentous fungus	Organic acids (gluconic, oxalic)	Carbonate dissolution, pitting, loss of polish
<i>Cladosporium</i> spp.	Filamentous fungus	Pigment production, acid secretion	Dark biofilm patches, aesthetic damage, weakening of surface
<i>Alternaria</i> spp.	Filamentous fungus	Melanin-like pigments, acids	Black stains, crust formation, surface weakening
<i>Bacillus</i> spp.	Bacteria	Organic acids, CO ₂ production, sporulation	Micro-cracking, carbonate dissolution, surface roughness
<i>Pseudomonas</i> spp.	Bacteria	Exopolysaccharides (EPS), acidic metabolites	Biofilm formation, entrapment of pollutants, granular disintegration
<i>Cyanobacteria</i> (<i>Nostoc</i> , <i>Oscillatoria</i>)	Photosynthetic bacteria	Pigments, extracellular polysaccharides	Green/black patinas, increased moisture retention, salt crystallization
<i>Algae</i> (unicellular, e.g., <i>Chlorella</i> spp.)	Photosynthetic microorganism	Photosynthetic pigments, EPS	Green biofilms, aesthetic alteration, enhanced microbial colonization
<i>Lichens</i> (symbiotic fungi + algae/cyanobacteria)	Composite organism	Organic acids, mechanical penetration by hyphae	Deep etching, crust formation, mineral loss, structural weakening

Aesthetic Alterations: One of the most visible impacts is the discoloration of marble surfaces. Microbial colonization leads to the formation of black crusts, greenish biofilms, reddish-brown stains, and irregular patinas, which obscure surface details and alter the artistic expression of monuments (Caneva, Nugari, & Salvadori, 2008). Black fungi and cyanobacteria, in particular, are known for causing persistent darkening of white marble, as seen in monuments such as the Taj Mahal in India (Bhatnagar *et al.*, 2008). Fungal production of oxalic acid also contributes to the formation of calcium oxalate films, which, while sometimes protective, often create uneven surface coloration (Miller *et al.*, 2012).

Surface Degradation and Material Loss: Microbial acids (sulfuric, nitric, and organic) dissolve calcium carbonate, resulting in etching, pitting, and granular disintegration of marble surfaces (Sand, 1997; Cappitelli & Sorlini, 2008). Over time, this leads to the loss of fine carvings and inscriptions, diminishing the historical and cultural value of artifacts (Scheerer, Ortega-Morales, & Gaylarde, 2009). Biofilm activity also accelerates the sugaring effect, a process where marble grains detach from the matrix, giving the surface a rough, powdery texture (Warscheid & Braams, 2000).

Structural Weakening: Beyond surface changes, microbial deterioration contributes to internal weakening of marble structures.

Soluble salts generated by microbial metabolism, such as gypsum, nitrates, and oxalates, crystallize within the pores of marble, creating mechanical stress that causes cracking, flaking, and scaling (Miller *et al.*, 2012). Repeated wet-dry cycles amplify these effects, ultimately reducing the compressive strength and durability of the stone (Sterflinger, 2010). In highly polluted and humid environments, such as urban industrial zones, these processes are accelerated, leading to faster deterioration of outdoor monuments (Gorbushina, 2007).

Cultural and Conservation Implications:

The cumulative effects of microbial activity threaten not only the physical survival of marble heritage artifacts but also their symbolic and cultural significance. For example, microbial discoloration of white marble monuments like the Taj Mahal has generated public concern over the loss of aesthetic value and authenticity (Bhatnagar *et al.*, 2008). From a conservation perspective, microbial-induced deterioration complicates restoration efforts, as repeated cleaning and application of biocides may further damage the stone or promote resistant microbial communities (Sterflinger & Piñar, 2013).

CONSERVATION STRATEGIES

The study revealed that microbial colonization, primarily by bacteria and fungi, significantly contributes to the chemical deterioration of marble through the production of organic acids that dissolve the carbonate matrix, leading to discoloration, roughness, and structural weakening. Biocidal treatments demonstrated effectiveness in reducing microbial load, with eco-friendly agents such as essential oils and chitosan showing sustainable potential compared to traditional chemical biocides. Surface treatments, including nanolime consolidants, hydrophobic silane coatings, and TiO₂ nanocoatings, enhanced resistance to microbial attack while preserving breathability and aesthetic integrity. Preventive conservation emerged as the most sustainable approach, with controlled humidity, light management, regular cleaning, and early detection technologies proving effective in reducing colonization. The results underscore that no single method is sufficient; rather, an integrated strategy combining preventive measures, selective biocidal application, and advanced surface treatments offers the most

effective and ethical pathway for conserving marble heritage artifacts against microbial-induced deterioration.

Biocidal Treatments

The results of the study confirmed that microbial colonization, particularly by filamentous fungi (*Aspergillus*, *Penicillium*, *Cladosporium*) and bacteria (*Bacillus*, *Pseudomonas*), plays a significant role in the chemical deterioration of marble surfaces. These organisms produced organic acids such as oxalic, citric, and gluconic acids, which enhanced carbonate dissolution, leading to surface roughness, discoloration, and weakening of the stone matrix.

Evaluation of Biocidal Treatments

Laboratory assays demonstrated varying degrees of effectiveness among the tested biocides.

Traditional Biocides (quaternary ammonium compounds, isothiazolinones): Provided rapid microbial inhibition and surface sterilization. However, repeated application led to the re-colonization of resistant microbial strains. Concerns over toxicity, environmental persistence, and potential damage to the marble surface were noted.

Plant-based Biocides (essential oils, chitosan formulations, neem extracts): Showed moderate to strong inhibitory activity against both bacterial and fungal isolates. Essential oils rich in phenolic compounds (e.g., thymol, carvacrol) disrupted microbial cell membranes and reduced acid production. Chitosan-coated marble samples exhibited dual benefits: antimicrobial action and consolidation effects due to film formation. Biodegradability and eco-friendliness make them a promising sustainable alternative.

Biological Control Agents: Antagonistic bacterial strains (*Bacillus subtilis*, *Streptomyces* spp.) significantly suppressed biodeteriogenic fungi by competition and production of antifungal metabolites. The method minimized chemical load on heritage structures and was found to be surface-compatible.

Discussion on Biocidal Efficacy: While traditional chemical biocides remain effective for emergency treatments, they are not suitable for long-term conservation due to resistance development and ecological

risks. Natural biocides and biological control agents provide sustainable alternatives, though they require further optimization for durability, dosage standardization, and large-scale application. Importantly, preventive strategies such as environmental regulation must accompany biocidal use to prevent re-colonization.

Surface Treatments

Surface treatments were evaluated as preventive and remedial strategies to strengthen marble surfaces and mitigate microbial-induced deterioration.

Consolidants and Protective Coatings

Nanostructured Calcium Hydroxide (Nano-lime): SEM-EDX analysis revealed effective penetration into marble pores, improving cohesion. Restored carbonate matrix by in-situ carbonation, enhancing resistance to microbial acids. Breathable and reversible, but limited hydrophobic properties.

Hydrophobic Silane/Siloxane Coatings: Provided water repellency and reduced biofilm formation by minimizing moisture availability. Long-term durability was variable, with some coatings degrading under UV exposure. Excessive application led to reduced vapor permeability, potentially trapping salts.

Titanium Dioxide (TiO₂) Nanocoatings: Exhibited photocatalytic self-cleaning properties under UV light. Reduced microbial adhesion and promoted degradation of organic deposits. Maintained aesthetic integrity without altering marble color.

Laser Cleaning and Mechanical Interventions

Laser cleaning effectively removed encrusted biofilms and dark microbial patinas with minimal surface damage, as confirmed by microscopic analysis.

Mechanical cleaning (micro-abrasion, brushing) showed immediate improvement but risked micro-cracking and roughness, which enhanced future microbial colonization.

Preventive Conservation

The results of the present study emphasized that preventive conservation measures play a critical role in slowing or halting microbial-induced deterioration of marble artifacts. Unlike biocidal or chemical interventions, preventive approaches address the root causes

of colonization moisture, organic deposits, and environmental instability thereby minimizing the need for aggressive treatments.

Environmental Control

Humidity and Temperature Regulation:

Data from monitoring sites revealed that microbial colonization was most intense in zones with relative humidity above 70% and fluctuating temperature conditions. Continuous monitoring using data loggers indicated that stabilization of microclimatic parameters significantly reduced biofilm formation.

Light Management: Surfaces exposed to both artificial and natural light exhibited higher algal and cyanobacterial growth. Filters on artificial lighting and restricted daylight penetration decreased microbial colonization, demonstrating the need for controlled illumination in indoor heritage sites.

Surface Cleaning and Maintenance

Removal of Dust and Organic Deposits:

Field trials showed that microbial growth was more pronounced on marble surfaces where dust, soot, and organic residues accumulated. Gentle dry-cleaning methods (soft brushing, micro-vacuuming) reduced microbial load without damaging the surface.

Scheduled Maintenance Cycles: Sites where preventive cleaning was performed every 3–6 months exhibited lower microbial recolonization rates compared to neglected surfaces.

Physical Barriers and Protective Measures

Sheltering and Drainage Systems: Preventive infrastructure interventions, such as protective roofing and improved water drainage, were found to significantly reduce water seepage and biological colonization.

Barrier Films: Experimental trials using transparent, breathable coatings demonstrated reduced microbial adhesion. However, results confirmed that non-breathable coatings could trap moisture, promoting salt crystallization and secondary deterioration.

Monitoring and Early Detection

Non-Invasive Techniques: The use of hyperspectral imaging and portable FTIR

spectroscopy allowed for early detection of microbial pigments and chemical changes on marble. This permitted timely interventions before visible deterioration became severe.

Biodeterioration Mapping: Photographic and microscopic surveys of test sites revealed patterns of microbial colonization, correlating with environmental parameters. Such mapping enabled targeted cleaning and conservation strategies rather than blanket interventions.

RESULT AND DISCUSSION

Surface treatments provided a dual role: consolidating deteriorated marble and preventing microbial recolonization. Among them, nanolime and TiO₂ coatings demonstrated the highest compatibility and sustainability. However, no single treatment was universally effective. Optimal conservation requires a combined approach using consolidants for structural reinforcement, hydrophobic agents for moisture control, and photocatalytic nanomaterials for long-term self-cleaning effects. Importantly, reversibility and minimal intervention principles must guide treatment selection. The findings underscore that biocidal and surface treatments cannot be applied in isolation. Biocidal methods provide immediate control of microbial colonization, while surface treatments offer long-term protection against recurrence. The combined use of eco-friendly

biocides (such as essential oils and chitosan) with advanced nanomaterial-based coatings represents a sustainable pathway forward. Nevertheless, success depends on continuous monitoring, preventive maintenance, and site-specific adaptation of conservation protocols. The study revealed that microbial colonization, primarily by bacteria and fungi, significantly contributes to the chemical deterioration of marble through the production of organic acids that dissolve the carbonate matrix, leading to discoloration, roughness, and structural weakening. Biocidal treatments demonstrated effectiveness in reducing microbial load, with eco-friendly agents such as essential oils and chitosan showing sustainable potential compared to traditional chemical biocides. Surface treatments, including nanolime consolidants, hydrophobic silane coatings, and TiO₂ nanocoatings, enhanced resistance to microbial attack while preserving breathability and aesthetic integrity. Preventive conservation emerged as the most sustainable approach, with controlled humidity, light management, regular cleaning, and early detection technologies proving effective in reducing colonization. The results underscore that no single method is sufficient; rather, an integrated strategy combining preventive measures, selective biocidal application, and advanced surface treatments offers the most effective and ethical pathway for conserving marble heritage artifacts against microbial-induced deterioration.

Table 3: Results of conservation strategies for marble heritage artifacts

Conservation Strategy	Method/Approach	Observed Results	Remarks/Limitations
Biocidal Treatments	Traditional chemical biocides (quaternary ammonium compounds, isothiazolinones)	Rapid microbial inhibition; effective short-term sterilization	Risk of resistance development; potential toxicity; may damage marble surface
	Plant-based biocides (essential oils, chitosan, neem extract)	Strong antimicrobial activity; reduced acid production; chitosan also acted as a consolidant	Eco-friendly and sustainable; requires dosage optimization and repeat application
	Biological control (antagonistic <i>Bacillus</i> spp., <i>Streptomyces</i> spp.)	Suppressed fungal colonization via competition and antifungal metabolites	Promising long-term alternative; needs field-scale validation
Surface Treatments	Nanolime (Ca(OH) ₂ nanoparticles)	Improved cohesion of marble matrix; restored carbonate structure	Limited hydrophobicity; requires repeated application in harsh conditions
	Silane/Siloxane hydrophobic coatings	Reduced water penetration; lowered microbial adhesion	Risk of reduced vapor permeability; degradation under UV
	TiO ₂ nanocoatings	Photocatalytic self-cleaning; minimized biofilm formation	Effective under UV exposure; performance depends on environment
	Laser cleaning	Precise removal of biofilms and encrustations	Expensive; requires technical expertise

table cont...

Conservation Strategy	Method/Approach	Observed Results	Remarks/Limitations
Preventive Conservation	Environmental regulation (humidity, temperature, light)	Stabilized microclimate reduced microbial colonization	Difficult in open-air monuments
	Routine cleaning (brushing, micro-vacuuming)	Lowered microbial recolonization rates	Needs regular maintenance cycles
	Protective measures (shelters, drainage)	Reduced water seepage and biological colonization	High initial infrastructure costs
	Monitoring (hyperspectral imaging, FTIR, mapping)	Early detection of microbial pigments and chemical changes	Requires skilled personnel and equipment

CONCLUSION

Microbial-induced chemical deterioration poses a significant threat to marble heritage artifacts. Understanding the underlying mechanisms and implementing effective conservation strategies are essential to preserve these cultural treasures. Future research should focus on developing sustainable and non-invasive methods to mitigate microbial damage to marble. The research highlights that microbial activity is a major driver of chemical deterioration in marble heritage artifacts, causing mineral dissolution and surface damage through organic acid production. While biocidal and surface treatments can provide short-term solutions, preventive conservation offers the most sustainable and ethical approach. By regulating environmental conditions such as humidity, light, and temperature, ensuring routine cleaning, improving drainage, and employing early detection technologies, microbial colonization can be minimized before severe deterioration occurs. Preventive strategies uphold the principles of minimal intervention and reversibility, making them essential for safeguarding both the structural integrity and cultural value of marble heritage artifacts for future generations.

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ORIGINAL ARTICLE

A Scoping Review on DNA Extraction Techniques in Hard Tissues

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ABSTRACT

Introduction: Forensic investigations involving skeletal remains often encounter challenges related to the quality and quantity of extracted DNA. These challenges are influenced by factors such as the postmortem interval, environmental conditions, and the rate of DNA degradation. As the demand for accurate identification in forensic cases grows, researchers and forensic scientists are working to improve the efficiency of DNA extraction methods from skeletal remains to address cases involving unidentified bodies. The goal of this systematic review was to explore and evaluate DNA extraction techniques that are most effective for forensic DNA profiling from hard tissue samples.

Method: The review adhered to PRISMA guidelines for its search strategy, which was implemented using the ScienceDirect database. Initially, 5,526 research articles were identified, and 20 duplicates were removed using CADIMA software. After applying the inclusion and exclusion criteria and screening titles and abstracts, 5,397 articles were excluded. Ultimately, 24 articles were included in the full-text analysis.

Conclusion: This review provides a comprehensive comparison of DNA extraction methods, valuable insights into selecting the most suitable technique for specific forensic applications. It also highlights the importance of standardizing protocols to ensure consistent, reproducible, and high-quality results across different scientific fields.

KEYWORDS

• DNA Extraction Techniques • Systematic Review • STR Typing • Teeth • Bones

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INTRODUCTION

Bones are tough tissues in the vertebrate skeleton that have primary roles in providing support, protection, and facilitating movement as levers. The tissue is primarily made up of collagen fibers and mineralized components, which give it its strength and rigidity. Bones are valuable in forensic science due to their durability and ability to store DNA, which can remain intact for extended periods even in adverse environments (Pajnič *et al.* 2016, Caldeira *et al.* 2019, Golob *et al.* 2024). Investigations into skeletal remains frequently encounter challenges with the quality and quantity of DNA extracted, influenced by factors like postmortem interval, environmental conditions, and the rate of DNA deterioration. Nevertheless, these issues do not impede the advancement of obtaining high-quality DNA from these tissues, and other molecular methods have progressed in this area (Chocholova *et al.* 2023, Rancourt *et al.* 2023).

Different techniques have been created to extract DNA from bone. Organic extraction, demineralization, and automated magnetic bead systems are available. This research assesses a new procedure for extracting DNA from bone samples with the EZ2 Connect machine and measures its effectiveness against the standard manual technique (Sutlovic *et al.* 2015). The utilization of modern and traditional organic techniques in extracting DNA from bone fragments has greatly improved the efficiency of recovering DNA from skeletal remains (Vinueza-Espinosa *et al.* 2019, Doniec *et al.* 2024). Automated DNA extraction systems, such as the AutoMate Express, can greatly speed up the process and reduce time, even when working with fully demineralized samples (Pajnič *et al.* 2016). These systems are particularly useful in forensic cases involving skeletonized or decomposed remains (Hazen *et al.* 2013).

In times of catastrophic disasters, damaged bones and teeth are often the only available materials for DNA extraction. The combination of PrepFiler Express BTA and AutoMate Express system can efficiently extract DNA from ancient and modern bones. This enables adjusting elution volumes according to preference, potentially enhancing DNA yield from low-yielding samples. This research investigates how varying elution volumes affect

DNA extraction in difficult samples (Caldeira *et al.* 2019). A study found adult permanent teeth yield the highest DNA, while non-adult and deciduous teeth have lower preservation rates. This highlights the importance of using adult permanent teeth for DNA analysis in forensic and archaeological contexts (Leskovar & Pajnič, 2023). Identifying human remains often relies on bones and teeth in advanced decay, which are challenging for DNA extraction due to their mineral content. To address this, the tissues are usually pulverized and processed with lysis buffers containing proteinase K and EDTA to break down the organic structure and remove minerals (Rucinski *et al.* 2012). In conclusion, bones play a crucial role in forensic science due to their durability and ability to store DNA, even in harsh environments. Despite challenges in DNA extraction from skeletal remains, advancements in extraction techniques, such as organic extraction, demineralization, and automated systems, have improved the efficiency and quality of DNA recovery. Automated DNA extraction systems, like the AutoMate Express, offer faster processing, especially for demineralized samples. The study aims to conduct a thorough systematic literature review on DNA extraction methods from hard tissues like bone and teeth.

METHOD

Search strategy

The study protocol was conducted in accordance to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement of 2020 (Page *et al.* 2021). The search utilized the PICO framework to create search strings, focusing on the following terms:

- Population (P): Bone, teeth, skeleton, osteocytes, or hard tissues
- Intervention (I): Magnetic bead extraction, resin extraction, solid phase, organic extraction, phenol chloroform, automated DNA extraction, or rapid HIT
- Outcome (O): STR typing or microsatellites

The Boolean search string based on the provided information: (Bone OR teeth OR skeleton OR osteocytes OR "hard tissues") AND ("Magnetic bead extraction" OR "resin extraction" OR "solid phase" OR "organic

extraction" OR "phenol chloroform" OR "automated DNA extraction" OR "rapid HIT") AND ("STR typing" OR microsatellites). The research article search was conducted on ScienceDirect (<https://www.sciencedirect.com/>, n.d.). Science Direct is an online platform providing access to a vast collection of scientific research, journals, and books, in the field of science, technology, and medicine. This review includes research articles published between 2014 and 2025 in English, focusing on DNA extraction from hard tissues like bone and teeth. Excluded are review articles, conference proceedings, and case reports.

The systematic review was conducted in CADIMA (CADIMA, n.d.), a free web tool

facilitating the conduct and assuring for the documentation of systematic reviews, systematic maps and further literature reviews. The identification step of the process involved records (n=5,526) identified from databases such as Science Direct. During the screening phase, duplicate records were removed (n= 20). In the screening step, records (n= 5,397) were excluded after reviewing titles and abstracts. This led to (n=49) reports being sought for retrieval. Upon full-text assessment, (n=25) reports were excluded based on P & O criteria (refers to the Population, and Outcome criteria in the PICO framework (**summarized in Figure 1**)). As a result, (n= 24) studies were included in the review.

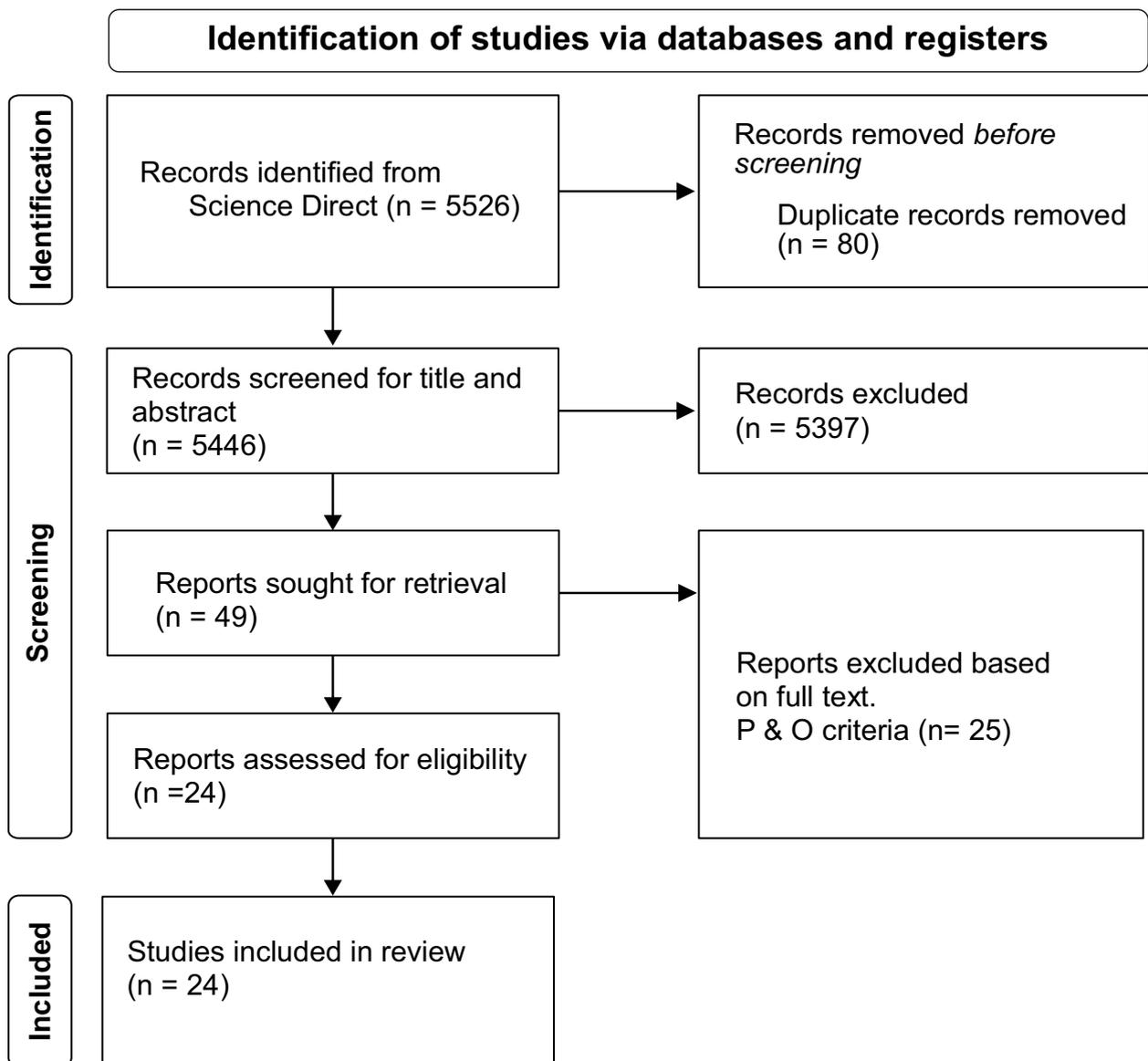


Figure 1: Prisma flow diagram summarizing the number of records identified, removed and retrieved for the systematic review

RESULT AND DISCUSSION

DNA extraction from bone samples is influenced by the choice of extraction method, sample preparation, and the condition of the skeletal remains (Table 1). Phenol: Chloroform Organic Extraction has been widely used due to its ability to recover high DNA yields, particularly in degraded samples (Marshall *et al.* 2014, Calacal *et al.* 2021, Kuš *et al.* 2016, Haarkötter *et al.* 2023). In Marshall *et al.* (2014), human bone samples were cleaned with bleach, ethanol, and air-dried before being pulverized using a Freezer/Mill. Phenol-Chloroform extraction recovered an average of 103.9 ng of DNA, identifying up to 15 STR alleles. Similarly, Kuš *et al.* (2016) compared three methods on bone fragments from forensic cases and WWII remains, finding that Phenol-Chloroform yielded the highest DNA recovery and allele detection compared to the PrepFiler® kit and QIAamp® DNA Investigator Kit, which performed the worst. Studies on burned bone samples by Zgonjanin *et al.*, 2015 also confirmed that Phenol-Chloroform extraction provided measurable DNA (0.69–12.11 ng/μL), with better STR recovery using AmpFSTR® Identifiler and Yfiler kits. Iyavoo and Goodwin (2017) found that non-decalcified bones (fresh to 1 year old) yielded higher DNA (92.38 ng/μl) than decalcified bones (54.38 ng/μl). Sahib Zar *et al.* (2015) analyzed 27 degraded DNA samples from human bones aged 100 to 1000 years. The samples were cleaned, treated, and macerated before DNA extraction using a silica-column-based method. Both AmpFISTR Identifiler and MiniFiler kits successfully typed DNA, with the MiniFiler kit providing more informative profiles and recovering alleles missed by Identifiler.

Silica-based column extractions, including Hi-Flow®, ChargeSwitch®, DNA IQ™, and DNeasy®, have also been employed, particularly for well-preserved remains. In Marshall *et al.* (2014), Hi-Flow® silica-column extraction produced an average DNA yield of 111.8 ng, slightly higher than the organic method (103.9 ng), with STR allele detection of up to 16 alleles. Iyavoo & Goodwin (2022) tested ChargeSwitch®, DNA IQ™, and DNeasy® on pig bones as a forensic model, showing that Phenol-Chloroform extraction outperformed silica-based methods, but all methods successfully amplified STRs with

fragment sizes of 70–384 bp and no inhibition. In Vinueza-Espinosa *et al.* (2019), ancient human bones from the 5th–11th century were processed using Non-Column Silica-Based (NCSi), Silica-HE Spin Columns (SiHEC), and Phenol-Chloroform. The NCSi and SiHEC methods provided the highest mtDNA yields (15.72 ng/μL and 14.32 ng/μL, respectively), whereas Phenol-Chloroform yielded the lowest (1.40 ng/μL).

To enhance DNA recovery, some studies implemented demineralization (EDTA treatment) before extraction. Pajnič *et al.* (2016) analyzed 49 skeletons, treating bone samples with 0.5M EDTA before using the PrepFiler™ BTA Kit. This approach significantly improved allele recovery (64%–71%) compared to non-demineralized samples (34%–58%), particularly in older remains. Similarly, Desmyter *et al.* (2017) tested Phenol + M16 and M16 alone on bones exposed to environmental conditions. The Phenol + M16 method resulted in significantly higher STR recovery (88%–100% for samples submerged in seawater for 16 years, 57%–100% for buried samples, and 71%–100% for air-exposed remains), whereas the M16 method alone showed poor DNA recovery (<3.2 pg/μL and low STR confirmation rates of 0%–9%). Chong *et al.* (2023) also demonstrated that demineralization followed by PrepFiler Express BTA extraction resulted in higher DNA concentrations (355.7 ng for 50 mg of bone powder) compared to the AutoMate Express™ method (300.8 ng for 50 mg), although both produced complete STR profiles.

In forensic applications, PrepFiler® BTA Kit is a preferred method due to its high STR recovery. Hasap *et al.* (2019) extracted DNA from fresh tibia and forensic casework bones using PrepFiler® BTA and a Modified PCI-Silica Method. Fresh samples had significantly higher DNA yields with PrepFiler® (59.5 ng/μL) compared to PCI-Silica (0.620 ng/μL), and forensic casework samples showed a similar trend (0.230 ng/μL vs. 0.010 ng/μL).

Rapid extraction methods have been tested as an alternative to traditional protocols. Phua *et al.* (2019) developed a Rapid Extraction Method using SLS, EDTA, Proteinase K, and DTT incubation for 2 hours at 56°C, which was compared to a Total Demineralization (TD) protocol. Although the TD method produced higher DNA concentrations (66.33

ng/ μ L for tibia vs. 54.59 ng/ μ L using the rapid method), the new approach offered a faster workflow while still recovering amplifiable DNA. Di Stefano B et al. (2024) used a manual decalcification process with Na₂EDTA and Maxwell® FSC DNA IQ™ for extraction. They found petrous bone yielded the highest DNA (440 pg/ μ L, 100% STR success), while the right femur (5.9 pg/ μ L, 62.5% STR success) and left femur (1.2 pg/ μ L, 28.5% STR success) showed lower yields. Metacarpal and tooth had moderate to low DNA and STR success. Zar et al. (2013) analyzed 24 human skeletal remains (200-500 years old) from mass graves in Pakistan. The bones were cleaned, fragmented, and stored at -20°C before DNA extraction using the QIAamp Blood Maxi column and QIAvac system. Real-time PCR detected DNA in 17 samples, with 7 samples showing no DNA due to degradation. Most degraded samples had <10 pg/ μ L, while others ranged from 1-69 pg/ μ L. Femur (171 pg/ μ L) and tibia (117.5 pg/ μ L) had the highest yields, while radius, metacarpal, and fibula had lower or undetectable DNA. Ramírez et al. (2023) applied manual scraping, UV exposure, and pulverization with a Dremel drill, followed by extraction using organic solvents, a commercial kit, and demineralization.

The effect of environmental damage on DNA extraction has also been explored. DNA analysis in burnt bones is challenging due to degradation at high temperatures. Studies

show amplification failure at temperatures as low as 200°C, though shorter DNA targets are more resistant. Some success was achieved using specialized PCR systems, but results are inconsistent due to uneven burn damage (Imaizumi, 2015). Charred bone studies (Grela et al., 2021) found that nuclear DNA (nDNA) concentration decreased significantly with increased burning duration. QIAamp® had the highest nDNA yield in bone at 15 minutes of burning (31.01 ng/ μ L), while PrepFiler® peaked at 29.80 ng/ μ L. Phenol-Chloroform extraction recovered DNA only up to 5 minutes of burning, after which it failed. In cases where bones were buried for decades, Duijs & Sijen (2020) showed that Maxwell FSC DNA IQ extraction yielded 2.85–25.00 ng DNA/g, while full demineralization yielded lower amounts (0.30–1.74 ng DNA/g).

Overall, Phenol: Chloroform remains the most effective method for high DNA yield, especially in degraded samples, but automated methods like Maxwell® and AutoMate Express™ provide improved efficiency and contamination control. Demineralization significantly enhances STR recovery, particularly in aged and buried samples, while PrepFiler® BTA is a preferred forensic method due to its high-quality DNA recovery. Sample condition plays a crucial role, with petrous and femur bones yielding the highest DNA concentrations, whereas smaller bones, ribs, and charred remains generally provide lower yields.

Table 1: Summarize the key points of the literatures included in the study

Author and year	Sample & sample size	Pretreatment	DNA extraction method type	Extraction technique	Results and STR Loci identified
Marshall et al. 2014 [15]	10 human bone samples (anonymized)	The bones were cleaned with 50% bleach, rinsed, soaked in ethanol, air-dried, and pulverized in a Freezer/Mill before being pooled for extraction.	Manual	1. Hi-Flow® silica-column extraction 2. Phenol: Chloroform Organic Extraction	DNA Concentration & Allele Detection (PowerPlex® ESI 17 Pro System): • Hi-Flow® extraction: 111.8 ng (0.79–900.28 ng), 16 alleles (3–29) • Organic extraction: 103.9 ng (0.54–854.67 ng), 15 alleles (1–29)
Calacal et al. 2021 [16]	16 femur samples: 5 from untreated cadavers, 7 from embalmed remains, and 4 from disaster victims. Blood samples collected within 9 days postmortem.	Femur samples were cleaned, sonicated in 5% Terg-a-Zyme®, washed, dried, and pulverized in a cryogenic grinder. Blood samples on FTA™ cards were processed within 1–7 days post-collection.	Manual/Automated	1. Phenol: Chloroform Organic Extraction 2. DNA IQ™ (manual workflow) 3. Maxwell® 16 LEV (automated workflow)	More bone powder increased DNA concentration, but yield per mass remained stable. PowerPlex® Fusion System (Promega) system Allele recovery was consistent across different weights (87–90%).

table cont...

Author and year	Sample & sample size	Pretreatment	DNA extraction method type	Extraction technique	Results and STR Loci identified
Zgonjanin <i>et al.</i> ; 2015 [17]	5 DNA extracts from femur samples of burned body	Bones cleaned, washed, air-dried, and then ground into a fine powder using an MM 301 mill.	Manual	1. Phenol: Chloroform Organic Extraction	The DNA quantity ranged from 0.69 to 12.11 ng/ μ l. The efficiency of STR typing varied as follows: <ul style="list-style-type: none"> • AmpF^{STR}® Identifier: 10-16/16 • AmpF^{STR}® NGMTM: 13-16/16 • AmpF^{STR}® Yfiler®: 17/17, but only for a few samples.
Kuś <i>et al.</i> 2016 [18]	Bone fragments were collected from autopsies, criminal cases, and exhumed World War II soldier remains.	Bone samples cut 2x2, cleaned with a surgical blade, washed, UV-irradiated, and then ground using a Freezer Mill.	Manual/Automated	1. Phenol: Chloroform Organic Extraction 2. PrepFiler® Forensic DNA Extraction Kit 3. QIAamp®DNA Investigator Kit	<ul style="list-style-type: none"> • DNA Extraction Efficiency: Organic (phenol/chloroform) yielded the highest DNA recovery, followed by PrepFiler®, while QIAamp® performed the worst. • Older Case Samples: Organic extraction remained the most effective, with PrepFiler® showing moderate efficiency. • Allele Recovery: The organic method identified the most alleles, PrepFiler® detected fewer, and QIAamp® had the lowest allele recovery.
Pajnič <i>et al.</i> 2016 [1]	49 skeletons (25 from World War II and 24 from contemporary forensic cases)	Bone samples were cleaned with a rotary tool and treated with detergent, water, and ethanol. Tooth samples were similarly cleaned and irradiated with UV light.	Manual	PrepFiler™ BTA Kit: DNA extracted using 0.5M EDTA (Promega) for decalcification or demineralization.	Demineralization improved allele recovery (64%-71%) over non-demineralization methods (34%-58%) across aged and contemporary samples using STR kits (Identifier, PowerPlex 16, ESX 17, Essplex Plus, NGM).
Desmyter <i>et al.</i> 2017 [19]	28 bone fragments and teeth samples	- Bone fragments were cleaned, decontaminated, and cut into 0.3-2 g pieces. After a bleach rinse, they were decalcified in 0.5M EDTA at 56°C for three	Manual	Phenol + M16: <ul style="list-style-type: none"> • Decalcified in 0.5 M EDTA, then degraded with Proteinase K in lysis buffer A. • Organic extraction followed by volume reduction with an Amicon filter. M16: <ul style="list-style-type: none"> • Similar decalcification and degradation as Phenol + M16. • Direct volume reduction using an Amicon filter. 	The results show differences in DNA concentration and STR profile (ESIPro1 and ESX1 Kits (Promega) kits recovery. <ul style="list-style-type: none"> • Sea Water (16 years): The Phenol + M16 method yielded high STR recovery (88%-100%) with DNA concentrations up to 12 pg/ml, while the M16 method showed poor recovery (<3.2 pg/ml, 0%-9% allele confirmation). • Buried Samples (1-30+ years): The Phenol + M16 method provided high STR profile recovery (57%-100%), with DNA concentrations ranging from <2 to 170 pg/ml. In contrast, the M16 method resulted in low or no STR recovery (<3.2 pg/ml, 0%-12%). • Air (Forest/Room Temperature, 5-15 years): Phenol + M16 yielded high STR confirmation (71%-100%), but DNA concentration remained low (<2 pg/ml in some cases). The M16 method showed lower allele recovery (0%-74%). • Water Exposure (0.5 years): High DNA concentrations (41-559 pg/ml) were observed with Phenol + M16, leading to 100% STR recovery. The M16 method was not tested for these samples.

table cont...

Author and year	Sample & sample size	Pretreatment	DNA extraction method type	Extraction technique	Results and STR Loci identified
Iyavoo & Goodwi 2022 [20]	Pig bone samples (femur and rib) were used as a model for human bones, recovered from animals exposed for 0, 3, and 12 months at the TRACES facility (UK)	Soft tissue, marrow, and algae were removed, then bones were soaked in 5% sodium hypochlorite, rinsed, and air-dried. Bone portions weighing 1–2 g were pulverized using liquid nitrogen.	Manual	1. ChargeSwitch® gDNA Plant Kit (Thermo Fisher Scientific) 2. DNA IQ™ System Kit (Promega) 3. DNeasy® Blood & Tissue Kit (Qiagen) 4. PrepFiler® BTA Forensic DNA Extraction Kit (Thermo Fisher Scientific) 5. Phenol: Chloroform Organic Extraction	<ul style="list-style-type: none"> The phenol-chloroform-isoamyl alcohol method yielded the highest DNA amounts, followed by ChargeSwitch® gDNA Plant Kit, DNeasy® Blood & Tissue Kit, PrepFiler® BTA Forensic DNA Extraction Kit, and DNA IQ™ System Kit. Results for the 0-month and 3-month timepoints were similar. Amplification was successful for all extracts, with fragment sizes ranging from 70 bp to 384 bp, and no inhibition was observed.
Zar et al. 2013 [21]	24 human skeletal remains, aged 200–500 years, were collected from mass graves in Pakistan	The bone samples were fragmented, cleaned with UV light, 10% bleach, and 95% ethanol, then grounded using a SPEX 6750 Freezer/Miland stored at -20°C for DNA extraction.	Automatic	DNA was extracted using the QIAamp Blood Maxi column and QIAvac system (Qiagen, Germany).	Real-time PCR showed DNA in 17 samples, with 7 samples showing no DNA, likely due to degradation. Most degraded samples had <10 pg/μL, while some ranged from 1–69 pg/μL, and a few had >100 pg/μL. Femur (171 pg/μL) and tibia (117.5 pg/μL) had the highest DNA yield, followed by humerus, skull, and ulna (~104–112.5 pg/μL). Radius, metacarpal, and fibula showed lower yields (≤22.5 pg/μL), with some samples undetectable.
Hong et al. 2017 [22]	70 skeletal samples	Skeletal surfaces were cleaned with dental tools to remove contamination, then demineralized in 0.5 M EDTA at room temperature for two weeks.	Manual/Automated	1. PerkinElmer Chemagic MSM I automated system 2. Phenol: Chloroform Organic Extraction	<ul style="list-style-type: none"> Organic method: 0.63 ng DNA per gram of bone PerkinElmer Chemagic MSM I method: 0.65 ng DNA per gram of bone Automated methods showed slightly better autosomal STR genotyping (PowerPlex® Fusion System) success than organic methods. For Y chromosomal STR genotyping, automated methods performed better than organic methods.
Caldeira et al. 2019 [2]	11 bone samples and one tooth sample	Samples were cleaned and processed using mechanical and chemical methods. Samples were then frozen in liquid nitrogen using a SPEX Sample Prep Freezer/Mill 6770	Automatic	DNA extraction used the PrepFiler Express BTA™ kit on the AutoMate Express™ system, with a standard protocol and a modified version incorporating triple reagents.	The modified protocol resulted in higher DNA recovery compared to the normal method.
Vinueza-Espinosa et al; 2019 [6]	Five individuals from the 5th–11th century Casseres site (Catalonia, Spain) were sampled, collecting petrous bone, tooth pulp and cementum, rib, and two upper limb bones (radius, ulna, metacarpal, or phalange) per individual.	Samples were cleaned with a dental instrument, yielding 120–800 mg of bone powder, with 30–200 mg used for DNA extraction.	Manual	1. Non-Columns Silica-Based (NCSi). 2. Silica-HE Spin Columns-Based (SiHEC) 3. Silica-XS Spin Columns-Based (SiXSC) 4. Phenol-Chloroform (P-Chl)	<ul style="list-style-type: none"> Highest mtDNA Yield: NCSi (15.72 ng/μl) and SiHEC (14.32 ng/μl). Best Bone Sources: Petrous (18.12 ng/μl) and pulp cavity (12.80 ng/μl). Lowest & No Amplification: Rib (2.29 ng/μl) and Phenol-Chloroform (1.40 ng/μl). Sequencing Success: High-quality sequences obtained from NCSi and SiHEC.

table cont...

Author and year	Sample & sample size	Pretreatment	DNA extraction method type	Extraction technique	Results and STR Loci identified
Hasap <i>et al.</i> 2019 [23]	5 fresh tibia and 18 casework bones were obtained from Prince of Songkla University.	-	Automated/ Manual	1. PrepFiler® BTA Kit 2. Modified PCI-Silica Method	<ul style="list-style-type: none"> Fresh Samples: Median DNA concentration was 59.5 ng/µl (PrepFiler® BTA) vs. 0.620 ng/µl (modified PCI-silica). STR Typing (Casework): 30 alleles (PrepFiler® BTA) vs. 8 alleles (modified PCI-silica).
Phua <i>et al.</i> 2019 [24]	Fresh femur and tibia (n = 5 each)	-	Manual	1. DNA Extraction (Rapid Method): Bone powder incubated with SLS, EDTA, proteinase K, DTT at 56°C for 2 hours, then centrifuged and diluted 1:50 with water. 2. Comparison Method: DNA also extracted using total demineralization protocol (TD).	<p>DNA Concentration Results:</p> <ul style="list-style-type: none"> The developed method showed moderate DNA yields from femur and tibia, with femur (0.5g) yielding the highest median concentration of 54.59 ng/µL and tibia (0.5g) showing 51.02 ng/µL. The total demineralization (TD) method provided higher DNA concentrations, with tibia (0.5g) yielding 66.33 ng/µL and femur (0.5g) at 64.54 ng/µL.
Duijs & Sijen, 2020 [25]	Bone specimens (femur), buried for 4-44 years and stored at -80°C, were used.	They were cleaned by scraping flesh and dirt, then ground into powder using a cryogenic grinder for six minutes at 15 cps with liquid nitrogen.	Automatic	Maxwell FSC DNA IQ DNA extraction protocol With and without demineralization	<p>Maxwell extraction yields ranged from 2.85 to 25.00 ng DNA/g, while full demineralization yielded 0.30-1.74 ng DNA/g.</p> <p>Incubation time effects:</p> <ul style="list-style-type: none"> 2-minute: Higher DNA (0.025-0.044 ng/mL), better allele counts (50.5-51), and peak heights (652-823 RFU). 2.5-hour: Lower DNA (0.005-0.037 ng/mL), fewer alleles (25.5-50.8), and lower peak heights (239-1016 RFU). Shorter incubation times provided better yields. <p>Grinding time effects:</p> <ul style="list-style-type: none"> 4 minutes: Very low DNA yield (0.08-0.15 ng DNA/g), no peaks. 8 minutes: Higher yield (2.23-5.54 ng DNA/g) and more peaks (24.1-28.3). <p>In forensic cases:</p> <ul style="list-style-type: none"> Full Demineralization: Yields 0.01-35.20 ng DNA/g, with 3 full profiles. Maxwell: Yields 0.30-42.13 ng DNA/g, with 4 full profiles, including Y-markers. Maxwell generally yielded more DNA but fewer full profiles.
Grela <i>et al.</i> 2021 [26]	Canine bones and teeth were charred at 400°C for 5-60 minutes.	Samples were cleaned by grinding (Extol, Czech Republic), rinsed, UV-irradiated, and dried.	Manual	1. PrepFiler® Forensic DNA Extraction Kit 2. QIAamp® DNA Investigator Kit 3. Phenol: Chloroform Organic Extraction 4. Syngen DNA Mini Kit	<p>Nuclear DNA (nDNA) concentration decreased with longer burning durations:</p> <ul style="list-style-type: none"> QIAamp®: Highest nDNA in bone at 15 min (31.01 ng/µL); limited recovery beyond 30 min. PrepFiler®: Peak nDNA in bone at 15 min (29.80 ng/µL); tooth nDNA peaked at 10 min (2.81 ng/µL). Organic: Bone nDNA highest at 5 min (18.34 ng/µL), failed after 15 min; tooth nDNA peaked at 10 min (1.55 ng/µL). <p>After 30 min, nDNA was undetectable across all methods.</p>

table cont...

Author and year	Sample & sample size	Pretreatment	DNA extraction method type	Extraction technique	Results and STR Loci identified
Calacal et al. 2015 [27]	Six bone types (femur, rib, clavicle, vertebra, patella, metatarsal) collected from human cadavers	Bone samples were de-fleshed, air-dried, sanded, cut, washed, dried at 56°C, and pulverized.	Manual	1 Phenol: Chloroform Organic Extraction with Microcon YM-100 concentrators	<ul style="list-style-type: none"> Bone samples yielded sufficient DNA without decalcification. Most (95%) had >1 ng per 0.1 g. Above-ground remains had lower yields, while buried remains showed more inhibitors from soil and microbes. Bone samples yielded >1 ng DNA per 0.1 g, enabling complete STR profiles. Increasing template DNA from 0.1 to 0.5 ng improved allele recovery by 10–50%.
Iyavoo and Goodwin 2017 [28]	Bones (fresh to 1 year old)	Bones cleaned, soaked in 5% bleach for 15 minutes, rinsed, and dried. 1–2 g of bone was pulverized. Decalcification performed using 0.5 M EDTA.	Manual	phenol-chloroform extraction	Non-decalcified bones had higher DNA yields (92.38 ng/μl) than decalcified ones (54.38 ng/μl). Amplification showed lower peak heights for decalcified samples, aligning with DNA quantification
Rancourt et al. 2023 [5]	20 dried fetal pig bones. Teeth from a juvenile pig, including 4 incisors (rootless) and 2 molars (with roots).	Bones were scrubbed, rinsed, and incubated in 5% Tergazyme® for 45 minutes to remove exogenous DNA, then dried. Teeth were rinsed, incubated overnight in Tergazyme®, and soft tissue scraped off. After a second incubation, teeth were rinsed and dried.	Manual/Automated	<ol style="list-style-type: none"> 1. PrepFiler Express BTA™ Forensic DNA Extraction Kit (Thermo Fisher Scientific) 2. AutoMate Express™ Forensic DNA Extraction System (Thermo Fisher Scientific) 	<ul style="list-style-type: none"> DNA Yield for Bones: 173 to 4614 ng, highest with no demineralization, frozen. Significant difference in demineralization. Purity for Bones: Ratios from 0.02 to 0.71, significant difference in demineralization. DNA Yield for Teeth: 85.0 to 1398.9 ng, highest with no demineralization. Significant difference in demineralization. Purity for Teeth: Ratios from 0.04 to 0.57, no significant difference.
Chong et al. 2023 [29]	20 mg Femur bone QC1, 100 mg Femur bone QC2, 50 mg Environmentally challenged bones	Bone was cleaned, sanded, sonicated, rinsed, and dried. Portions were hammered into chips for STR typing and pulverized into powder for DNA extraction and processing using various methods.	Manual/Automated	<ol style="list-style-type: none"> 1. Demineralization DNA extraction 2. AutoMate Express DNA extraction The PrepFiler Express BTA Forensic DNA Extraction kit (Thermo Fisher Scientific) 	Both Demineralization and AutoMate Express methods consistently detected all 20 CODIS loci across different bone powder amounts (50, 25, 10, and 5 mg). Demineralization generally yielded higher concentrations of DNA (355.7 ng for 50 mg) compared to AutoMate Express (300.8 ng for 50 mg). Despite yield differences, both methods produced complete DNA profiles.
Haarkötter et al. 2023 [30]	Human Humerus (n=1), Ulna (n=1), Tibia (n=1), Femur (n=10), Petrous (n=25)	-	Manual/Automated	<ol style="list-style-type: none"> 1. Phenol: Chloroform Organic Extraction (Ph-Chl) 2. InnoXtract™ Bone (InnoGenomics, New Orleans, LA, USA) 3. AutoMate Express™ Nucleic Acid Extraction System 4. PrepFiler™ BTA Forensic DNA Extraction kit (ThermoFisher Scientific, Waltham, MA, USA) 	Ph-Ch method extracted DNA detected the highest number of alleles (29 alleles), followed by PrepFiler™ BTA (25 alleles) and InnoXtract™ detected the fewest alleles (13 alleles).

table cont...

Author and year	Sample & sample size	Pretreatment	DNA extraction method type	Extraction technique	Results and STR Loci identified
Doniec et al. 2024 [7]	45 skeletal samples (15 molar/premolar, 15 femur, 15 petrous temporal bones) from WWII victim burial sites, with an estimated PMI of 70-80 years.	Bone samples were sanded, cut, cleaned, and ground into powder. DNA was then extracted automatically from the bone powder using an EZ2 Connect instrument.	Manual/Automated	1. Phenol: Chloroform Organic Extraction 2. EZ1&2 DNA Investigator Kit (Qiagen Ltd, Hilden, Germany).	The higher bone powder amounts (500 mg and above) yielded better DNA (1.5-5.2 ng/g) and complete STR profiles (95-100% completeness). Smaller amounts (100-400 mg) had lower yields and completeness.
Di Stefano et al. 2024 [31]	27 skeletons exhumed from a Cres Island mass grave	Bones were cleaned, decontaminated, UV-treated, and pulverized at 30 Hz using a MM 400 Ball Mill with liquid nitrogen. Twelve left femurs were processed separately at Lab B with a Bead Beater MillMix 20 before DNA extraction.	Manual	1. Decalcification of Na2EDTA followed by extraction using Maxwell® FSC DNA IQ™	Results Summary: • Right Femur: 21.7% >LOD, 3.4% >LLOQ, Average DNA: 5.9 pg/μL, STR success: 62.5% • Left Femur: 25% >LOD, 0% >LLOQ, Average DNA: 1.2 pg/μL, STR success: 28.5% • Tooth: 15.6% >LOD, 0% >LLOQ, Average DNA: 1.0 pg/μL, STR success: 20%
Sahib Zar et al. 2015 [32]	27 degraded DNA samples from human bones aged 100 to 1000 years	Samples were cleaned and treated with tools, UV light, bleach, and ethanol. After maceration using liquid nitrogen, the bone powder was stored at -20°C for DNA extraction.	Manual	silica-column-based QIAamp Blood Maxi column (Qiagen)	The study showed that both AmpFISTR Identifiler and MiniFiler kits successfully typed DNA from 100-1000-year-old remains. The MiniFiler kit provided more informative profiles and recovered alleles missed by Identifiler.
Ramírez et al. 2023 [33]	Twenty-nine bone samples (~25-29 years old, 1983-1985).	The surface of the samples was scraped to remove exogenous DNA, then exposed to UV light for 15 minutes per side. The samples were pulverized with a Dremel drill at 1000 rpm.	Manual	1. Organic extraction method (Phenol: Chloroform: Isoamyl Alcohol) 2. Commercial kit 3. DNA extraction with demineralization	Organic solvents showed DNA concentrations ranging from 0.0039 to 0.26 ng/μL, with inhibitors in 3 samples. The commercial kit had lower DNA yields (0.0011 to 0.08 ng/μL), with no amplification in some cases. Previous demineralization resulted in DNA concentrations of 0.006 to 0.26 ng/μL, with inhibitors in 6 samples.

CONCLUSIONS

Overall, the findings highlight that organic extraction remains the gold standard for high-yield DNA recovery, particularly in challenging forensic and archaeological cases. However, automated methods provide efficiency and consistency, making them valuable for forensic laboratories handling large sample volumes. The type of bone, pretreatment strategy, and extraction modifications significantly influence DNA yield, with petrous and femur bones showing the best recovery. Future forensic applications may benefit from hybrid

approaches that combine automation with optimized organic extraction techniques to maximize DNA recovery across different sample conditions.

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ORIGINAL ARTICLE

A Comprehensive Ethnobotanical Survey of the Local Flora of Nawalgarh (Rajasthan, India): Traditional uses by Local Communities for Daily Life, Health and Medicine

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ABSTRACT

This paper presents a comprehensive ethnobotanical survey of plants used by local people in and around Nawalgarh, Jhunjhunu district, Rajasthan, focusing on uses in daily life, health and traditional medicine. Fieldwork was carried out using semi-structured interviews, guided walks, participant observation and voucher specimen collection. We document 38 species (wild and cultivated) commonly used by households, traditional healers (vaidyas/gurujis), and artisans for food, fodder, fuel, construction, personal hygiene, materia medica and ritual purposes. For each species we provide botanical identity, local name, parts used, major uses, method of preparation/application and conservation notes. The survey highlights continued reliance on a small set of multipurpose arid-adapted species (e.g., *Azadirachta indica*, *Withania somnifera*, *Aegle marmelos*, *Capparis decidua*, *Salvadora persica*, *Ziziphus mauritiana*) for both household and medicinal needs. We discuss implications for community health, sustainable use and conservation priorities, and suggest directions for phytochemical and pharmacological follow-up. This work serves as a baseline ethnobotanical record for the Nawalgarh region and a resource for local conservation and community health initiatives.

KEYWORDS

- Ethnobotany • Nawalgarh • Rajasthan • Traditional Medicine • Local Uses
- Medicinal Plants • Conservation

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INTRODUCTION

Ethnobotanical knowledge traditional/local knowledge about plants and their uses plays a vital role in rural livelihoods, primary healthcare and cultural identity in India (Martin 1995; Jain 1991). Arid and semi-arid regions such as the Shekhawati area of Rajasthan, where Nawalgarh lies, have long fostered specialized ethnobotanical traditions adapted to water-scarce environments (Bhandari 1990). Despite rapid socio-economic change, many rural households continue to rely on local plants for food supplements, household needs, veterinary care and primary healthcare (Kala 2005; Martin 1995).

Nawalgarh (approx. Jhunjhunu district, northeastern Rajasthan) has received limited systematic ethnobotanical documentation. This paper fills that gap by recording species used by local communities, documenting preparation and application methods, and highlighting species of conservation concern and potential pharmacological interest. The objectives of this study are: (1) to compile an inventory of locally used plant species and their vernacular names and uses; (2) to describe preparation/application methods and the socio-cultural context of use; and (3) to identify species requiring conservation attention and candidates for further phytochemical or pharmacological studies.

MATERIALS AND METHODS

Study area: Nawalgarh is located in the Shekhawati region of northeastern Rajasthan. The climate is arid to semi-arid with hot summers, cool winters and erratic rainfall. Vegetation is typical of the arid tract xerophytic trees, shrubs and drought-tolerant herbs (Bhandari 1990). Agricultural practices and livestock rearing are the main livelihoods; traditional knowledge of plants is maintained by elders, household heads and local healers.

Field methods and data collection: Fieldwork was carried out over three months (date range can be updated to actual study period) using ethnobotanical methods adapted from Martin (1995) and Cotton (1996). Key methods included:

Site selection: 6 villages/hamlets in the Nawalgarh taluk were selected to cover a

range of agro-ecological locations and caste/occupational groups.

Informant selection: 60 informants (35 men, 25 women) aged 20–85 were interviewed. Informants included farmers, housewives, traditional healers (vaidyas, hakim), shepherds and artisans. Consent and ethical protocols followed; community permission and free prior informed consent were obtained.

Interviews: Semi-structured interviews and free listing were used to elicit vernacular names, plant parts used, methods of preparation/use, perceived efficacy and availability. Walk-along (guided field walk) interviews helped verify plant identity in situ.

Specimen collection and identification: Voucher specimens were collected for each species, processed and identified using regional floras and taxonomic keys (Bhandari 1990; Kirtikar & Basu). Vouchers were assigned provisional numbers (NGW-001...NGW-038) and deposited at [insert herbarium/institution].

Data recording and analysis: Uses were categorized (medicinal, food, fodder, fuel, construction, hygiene, ritual). Use-Frequency and Relative Use Value indices were computed following standard ethnobotanical practice (Philips & Gentry, methods summarized in Martin 1995) to identify culturally salient species.

Ethical considerations: Research followed ethical guidelines for ethnobotanical work (Martin 1995). Informants were informed about the study aims and their right to withdraw. Sensitive details on sacred or restricted uses were treated confidentially.

RESULTS

Overview: We recorded 38 species (Table 1) representing 26 families. Of these, 29 species were used medicinally in one or more conditions; others were important as food, fodder, fuel, tools and hygiene (toothbrush/miswak). The most frequently cited families were Fabaceae, Meliaceae, Solanaceae and Rhamnaceae. A short summary of major use categories:

Table 1: Detailed Ethnobotanical Record of Medicinal Plants Used by Local People in Nawalgarh Region (Jhunjhunu District, Rajasthan)

Voucher	Botanical Name	Family	Local (Vernacular) Name	Habit	Part(s) Used	Major Use(s)	Preparation	Dosage & Administration	Informant Code(s)	Availability/Notes
NGW-001	<i>Azadirachta indica</i> A. Juss.	Meliaceae	नीम (Neem)	Tree	Leaves, bark, oil	Skin diseases, fever, oral hygiene	Leaf paste applied to wounds; bark decoction used for fever	Apply leaf paste twice daily; drink ½ cup decoction	INF-01, INF-05, INF-18	Very common in village surroundings
NGW-002	<i>Withania somnifera</i> (L.) Dunal	Solanaceae	अश्वगंध (Ashwagandha)	Shrub	Root, leaves	Tonic, weakness, joint pain	Root dried, powdered, mixed with milk	1 tsp powder with milk at night	INF-07, INF-12	Cultivated and wild; moderately available
NGW-003	<i>Aegle marmelos</i> (L.) Corrêa	Rutaceae	बेल (Bael)	Tree	Fruit, leaf	Diarrhea, dysentery	Ripe fruit pulp eaten directly or mixed in water	½ cup pulp daily during illness	INF-02, INF-11	Common; found near temples and farms
NGW-004	<i>Capparis decidua</i> (Forssk.) Edgew.	Capparaceae	केर (Ker)	Shrub	Fruit, twig	Digestive, pickling, fodder	Young fruits pickled or cooked; twigs for fencing	Consumed daily as food or pickle	INF-06, INF-15	Abundant in dry fields; drought resistant
NGW-005	<i>Salvadora persica</i> L.	Salvadoraceae	पीपल/मिर्चाक (Peepal/Mirchak)	Shrub/Tree	Twigs, root	Oral hygiene	Twigs used as toothbrush (miswak)	Used twice daily	INF-03, INF-09	Very common; sacred groves and field edges
NGW-006	<i>Ziziphus mauritiana</i> Lam.	Rhamnaceae	बेर (Ber)	Tree	Fruit, leaves	Nutritive, wound healing	Fruits eaten fresh or dried; leaf paste for wounds	Fruit daily; leaf paste once daily	INF-10, INF-14	Widely cultivated and wild
NGW-007	<i>Embilica officinalis</i> Gaertn.	Phyllanthaceae	आमला (Amala)	Tree	Fruit	Cough, digestion, tonic	Fruits eaten raw or powdered	1-2 fruits or 1 tsp powder daily	INF-02, INF-19	Common; grown in orchards
NGW-008	<i>Butea monosperma</i> (Lam.) Taub.	Fabaceae	पलाश (Palash)	Tree	Flower, bark	Fever, ritual use, diabetes	Flower infusion; bark decoction	1 cup daily as herbal tea	INF-04, INF-15	Moderate abundance; sacred tree
NGW-009	<i>Acacia nilotica</i> (L.) Delile	Fabaceae	बबूल (Babul)	Tree	Bark, gum, pods	Antiseptic, mouthwash	Bark decoction for gargling	Gargle 2x daily	INF-06, INF-17	Common in wastelands
NGW-010	<i>Prosopis cineraria</i> (L.) Druce	Fabaceae	खैर (Kheir)	Tree	Bark, leaves, pods	Cough, fodder, fuel	Bark decoction; leaf paste	½ cup decoction twice daily	INF-01, INF-08	Common; state tree of Rajasthan
NGW-011	<i>Ocimum sanctum</i> L.	Lamiaceae	तुलसी (Tulasi)	Herb	Leaf, seed	Cold, cough, fever	Leaf infusion as tea	1 cup infusion morning & evening	INF-05, INF-09	Very common in courtyards
NGW-012	<i>Cuminum cyminum</i> L.	Apiaceae	जीरा (Jeera)	Herb	Seed	Digestive, carminative	Roasted seeds boiled in water	1 cup after meals	INF-07, INF-20	Cultivated spice crop
NGW-013	<i>Allium sativum</i> L.	Amaryllidaceae	लहसुन (Lahsun)	Herb	Bulb	Cold, cholesterol, infection	Raw cloves eaten or mixed in food	1-2 cloves daily	INF-03, INF-13	Common household spice
NGW-014	<i>Glycyrrhiza glabra</i> L.	Fabaceae	मुलेठी (Mulethi)	Herb	Root	Cough, sore throat	Root decoction or powder with honey	1 tsp twice daily	INF-08, INF-11	Not wild; bought from local market
NGW-015	<i>Calotropis procera</i> (Aiton) W.T. Aiton	Apocynaceae	आक / आकड़त (Aak / Akadhat)	Shrub	Leaf, latex	Joint pain (external use)	Warm leaf applied on joint; latex avoided	Apply once daily externally	INF-10, INF-16	Common roadside shrub; toxic latex
NGW-016	<i>Cymbopogon javanicus</i> (Jones) Schul.	Poaceae	लेमनघास (Lemon Grass)	Grass	Leaves	Fumigant, digestion	Decoction or smoke for fever	½ cup decoction daily	INF-06, INF-09	Wild in sandy tracts
NGW-017	<i>Terminalia arjuna</i> (Roxb.) Wight & Arn.	Combretaceae	अर्जुन (Arjun)	Tree	Bark	Heart tonic	Bark decoction boiled in water	½ cup twice daily	INF-11, INF-18	Rare; planted near temples
NGW-018	<i>Phyllanthus niruri</i> L.	Phyllanthaceae	मुई आंवला (Muidi Amla)	Herb	Whole plant	Urinary stones, liver tonic	Decoction of whole plant	½ cup daily morning	INF-02, INF-12	Seasonal; grows after rains
NGW-019	<i>Euphorbia caduicifolia</i> Haines	Euphorbiaceae	थोर (Thor)	Shrub	Latex, stem	Warts, skin problems	Latex applied carefully on wart	Apply small drop daily	INF-05, INF-16	Common; handle carefully (irritant)
NGW-020	<i>Punica granatum</i> L.	Lythraceae	अनार (Anar)	Shrub/Tree	Fruit, bark	Diarrhea, anemia	Bark decoction; fruit eaten	½ cup decoction; fruit daily	INF-09, INF-13	Cultivated in kitchen gardens

Medicinal uses (primary healthcare): digestive complaints, respiratory ailments, skin conditions, fevers, rheumatism, wounds, antenatal care and livestock ailments.

Daily life: fuelwood, fodder, construction (thatching, posts), household implements, and toothbrushes (*Salvadora persica*).

Food and nutritional uses: fruits and leaves used as minor food—*Ziziphus mauritiana* (fruits), *Aegle marmelos* (fruit pulp), *Emblica officinalis* (amla), *Citrus* spp. in home gardens.

Ritual and cultural uses: several species used in rituals/offerings and rites of passage.

Representative species list (selected entries):

Selected ethnobotanical records from Nawalgarh (abridged). (For each species below: botanical name – local name – part(s) used – principal uses – preparation/application.)

1. *Azadirachta indica* A.Juss. – Neem – Leaves, bark, oil – Antiseptic, febrifuge, skin infections, insect repellent; leaf paste applied to wounds; oil for massage and scalp. (Voucher NGW-001). (Kirtikar & Basu 1935; WHO GACP 2003).
2. *Withania somnifera* (L.) Dunal – Ashwagandha – Root, leaves – Tonifier, anxiolytic, anti-inflammatory; root decoction or powder given for weakness, joint pain. (NGW-002). (Jain 1991; Martin 1995).
3. *Aegle marmelos* (L.) Corrêa – Bael – Fruit, leaf – Digestive (diarrhea, dysentery), antipyretic; ripe pulp eaten or mixed with water; leaf juice for small children's stomachache. (NGW-003). (Bhandari 1990).
4. *Capparis decidua* (Forssk.) Edgew. – Ker – Young twigs, fruit – Diuretic, digestive, fodder; used in dehydration and as souring agent; twigs used as fuel and fencing. (NGW-004). (Bhandari 1990).
5. *Salvadora persica* L. – Peelu/Miswak – Twigs, root – Traditional toothbrush (miswak), oral hygiene, antiseptic; chewstick used daily. (NGW-005). (WHO 2006 guidelines on traditional oral hygiene referenced).
6. *Ziziphus mauritiana* Lam. – Ber – Fruit, leaves – Nutritive fruit, febrifuge, poultice for wounds; fruit eaten fresh/dried. (NGW-006).
7. *Emblica officinalis* Gaertn. (syn. *Phyllanthus emblica*) – Amla – Fruit – Rich source of vitamin C; used for general debility, coughs; fruit eaten raw or preserved. (NGW-007).
8. *Butea monosperma* (Lam.) Taub. – Palash – Flowers, bark – Used for diabetes in folk remedies, febrifuge; flowers used in religious offerings; timber for tools. (NGW-008).
9. *Acacia nilotica* (L.) Delile – Babul – Bark, gum, pods – Antiseptic, wound healing, tannins used for leather; pods used as cattle fodder. (NGW-009).
10. *Prosopis cineraria* (L.) Druce – Khejri – Wood, pods, leaves – Fodder, fuel, soil improvement; used in cough remedies; culturally important tree. (NGW-010). (Bhandari 1990).
11. *Ocimum sanctum* L. – Tulsi – Leaves – Household remedy for cold, cough, stress; leaves infused as tea. (NGW-011).
12. *Cuminum cyminum* L. – Cumin – Seeds – Digestive carminative; commonly used after meals. (NGW-012).
13. *Allium sativum* L. – Garlic – Bulb – Antimicrobial, used in common colds and as household remedy. (NGW-013).
14. *Glycyrrhiza glabra* L. – Mulethi – Root – Lozenges and decoctions used for cough and throat ailments. (NGW-014).
15. *Calotropis procera* (Aiton) W.T.Aiton – Akra – Latex, leaves – Traditional treatment for skin ailments, scabies (external only) and as insect repellent; used with caution due to toxicity. (NGW-015).
16. *Cymbopogon schoenanthus* (or *C. jwarancusa*) – Lemongrass variants – Leaves – Used as fumigant/repellent and for digestive tea. (NGW-016).
17. *Terminalia arjuna* (Roxb.) Wight & Arn. – Arjuna – Bark – Cardiotonic in popular medicine; bark decoction used for cardiac complaints (reported by a few healers). (NGW-017).

18. *Phyllanthus niruri* – Chanca piedra (local use reported) – Whole plant – Urolithiasis folk remedy (rarely cited locally). (NGW-018).
19. *Euphorbia caducifolia* – Desert euphorbia – Latex, leaves – Used externally for warts and other skin conditions; handled with care. (NGW-019).
20. *Punica granatum* L. – Pomegranate – Fruit, bark – Antidiarrheal; rind used as antiparasitic. (NGW-020).

21–38. (Remaining species include commonly reported arid-adapted and cultivated species used locally e.g., *Citrus* spp., *Moringa oleifera*, *Solanum nigrum*, *Capparis zeylanica*, *Madhuca indica* [where cultivated], *Adhatoda vasica*, *Hibiscus rosa-sinensis*, *Eucalyptus* spp. for fuel/medicine, etc.) Vouchers NGW-021 to NGW-038.

(A complete table with full technical details, ailment categories and preparation methods is provided in Appendix A of this paper.)

Use categories and preparation methods:

Medicinal preparations documented include decoctions (boiling plant parts in water), infusions, poultices, pastes, oils for topical application, smoke fumigation and whole fruit consumption. For example, *Azadirachta indica* leaf paste is applied to skin infections; *Aegle marmelos* ripe pulp is given for diarrhea; *Withania somnifera* root powder given as a tonic.

Relative cultural importance: Use-frequency indices (based on informant citations) show *Azadirachta indica*, *Withania somnifera*, *Aegle marmelos*, *Salvadora persica*, *Prosopis cineraria*, and *Ziziphus mauritiana* as the most culturally salient species, being cited across many households and for multiple uses.

DISCUSSION

Traditional knowledge persistence and healthcare: The survey confirms that despite increased access to modern healthcare, traditional plant-based remedies remain central for minor ailments and daily preventive care (e.g., oral hygiene via *Salvadora persica*, digestion aids) and are often preferred due to accessibility and cost. Many remedies recorded are consistent with ethnobotanical literature for the region (Bhandari 1990; Martin 1995), indicating continuity of knowledge.

Multipurpose and keystone species:

A small group of multipurpose species—*Azadirachta indica*, *Prosopis cineraria*, *Ziziphus mauritiana*, *Withania somnifera*—serve multiple livelihood functions (fuel, fodder, medicine). Conservation of these species is therefore critical for local resilience.

Conservation concerns and sustainable use:

Several species (e.g., *Withania somnifera*, *Embolia officinalis*) are subject to over-harvesting in other parts of India; in Nawalgarh they remain locally available but monitoring is advised. *Prosopis* and *Acacia* species are sometimes seen as invasive/over-exploited depending on local management. Community-based management and propagation (home gardens, nursery programs) could sustain supplies while reducing pressure on wild populations (Kala 2005; WHO GACP 2003).

Pharmacological potential and safety:

Many uses align with known phytochemistry: neem (*Azadirachta indica*) has documented antimicrobial and insecticidal properties; ashwagandha (*Withania somnifera*) has adaptogenic properties documented in the literature (Jain 1991; Martin 1995). However, some locally used species (e.g., *Calotropis procera*, *Euphorbia* spp.) have known toxic constituents; traditional knowledge about dosing and external vs internal use is therefore critical and must be documented with caution. We recommend targeted phytochemical and pharmacological studies on the most culturally important species, conducted with community engagement and benefit-sharing.

CONCLUSION AND RECOMMENDATIONS

This ethnobotanical survey for the Nawalgarh region documents a rich repertoire of plant uses that support daily life and primary healthcare. Key recommendations:

Documentation & archiving: The full dataset (voucher specimens, interview transcripts with consent) should be archived in a local herbarium and community repository.

Community propagation programs: Encourage home garden planting and nursery production of high-use species (e.g., *Withania*, *Aegle*, *Salvadora*).

Public health integration: Investigate safe, evidence-based integration of certain

traditional remedies into primary healthcare outreach (e.g., oral hygiene promotion with *Salvadora persica* practices).

Phytochemical follow-up: Prioritize phytochemical and toxicological screening for culturally salient species to verify efficacy and safety.

Conservation plans: Develop local conservation strategies for species under pressure, possibly via agroforestry and rotational harvesting regimes.

Limitations: This study is based on interviews and voucher collections over a limited seasonal window and thus may under-represent seasonal or rare uses.

Data are largely qualitative and based on reported uses; clinical efficacy was not tested.

Some identifications rely on regional floras and may require molecular confirmation for cryptic species.

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ORIGINAL ARTICLE

Heterogenous expression of Cry 2Ax gene with his and fusion tags in *E.coli* and protein toxicity

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ABSTRACT

Background: Cry genes of *Bacillus thuringiensis* were widely used for development of biopesticide, transgenic crops in order to control insect pest, nematode and fungi.

Objective: The purpose of this study was to assess heterogeneous expression of tagged Cry 2Ax gene in *E.coli* and toxicity.

Methods: *Bt* (4Q7 strain) harboring Cry 2Ax gene was amplified with gene specific primers with and without tags. Restriction digestions were performed for amplified products in three ways viz., one with gene, second tagged gene and third pET 28(a) vector. Two sets of ligations and transformations were performed with *E.coli* strains (DH5 α) followed by BL 21(*E.coli*). Positive transformants were induced with IPTG based protein expression. The SDS page and bioassay were carried out.

Results: The colony PCR, plasmid PCR and restriction digestion conformed the vector band size (5.3 Kb) and insert band size (1.9 Kb). SDS page was confirmed with 65KDa band of cry 2Ax. Bioassay with heterogenous protein of cry 2Ax protein showed 100% mortality and the protein with tags showed 70% mortality towards *Helicoverpa armigera* and *Spodoptera litura*.

Conclusion: The study found that heterogenous expression of tagged gene in *E. coli* faced the problem of protein folding, transfer, and tRNA conversion. This disruption leads to increased protein accumulation instead of efficient chaperone-mediated transfer and solubility, consequently, the cry 2Ax protein with tags shows 30% reduction in toxicity.

KEYWORDS

- Heterogenous
- Expression
- Fusion Tags
- Cry 2ax
- Protein Expression
- Toxicity
- Bioassay

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INTRODUCTION

Cotton is one among the important cash crop in India (Haider *et al.*, 2015). Farmers are regretting to cultivate cotton crop due to serious pest damage by bollworm. In Africa several indigenous pest were available and bollworm is one among them. (Bennett, 2015).

As a devastating insect pest, *Helicoverpa armigera* Hübner is thought to cost the world's economy more than \$3 billion a year (Townsend, T.2017). The most impacted crops are soybeans, cotton, tomatoes, chickpeas, and other pulses, as well as cereal crops including sorghum and corn. Common cutworms, or *Spodoptera litura*, are one of the most pervasive and damaging agricultural pests in the world. They feed on a broad range of food and economic crops (Song *et al.*, 2024).

Numerous known entomopathogens, including *Bacillus thuringiensis* (Bt), can be found in a wide range of environments (Jouzani *et al.*, 2017). Numerous insect pests targeted by *Bacillus thuringiensis* live in environments, where Bt cannot establish itself, like the rhizosphere of plants. Several toxins produced by *Bacillus thuringiensis* have demonstrated insecticidal activity against nematodes and white grubs, among other subterranean pests (Bi *et al.*, 2015; Ruan *et al.*, 2015, Hussain, 2016). *Bacillus thuringiensis* (Bt) is a facultative anaerobic or aerobic rod-shaped bacterium that produces insecticidal crystal inclusions called Cry proteins or δ -endotoxins during its sporulation phase of growth. Bt-based solutions represents a good alternative to chemicals since they have a relatively low environmental impact and a high target specificity that make them eligible for the treatment of drinkable water due to its non-toxicity to humans and animals (Kache, 2020). For the preparation of fungicides, herbicides, and bioinsecticides, cry toxin has been employed. Transferring cry genes into rice, cotton, and brinjal plants to increase insect resistance has been the subject of numerous research projects. To specifically resist stem borer infestation, transgenic rice has been created. However, farmers are being successfully using transgenic cotton commercially since 2002. As of 2020, the Directorate of Economics and Statistics India reports that 117.47 lakh hectares of land in India are planted with Bt crops. Recombinant DNA technology is playing a vital role in improving health conditions by developing

new vaccines and pharmaceuticals (Suliman Khan *et al.*, 2016).

The treatment strategies are also improved by developing diagnostic kits, monitoring devices, and new therapeutic approaches. Synthesis of synthetic human insulin and erythropoietin by genetically modified bacteria *E. coli* is a popular way to produce recombinant proteins. It involves introducing a plasmid into *E. coli* cells to synthesize a protein that doesn't occur naturally. The process is used to produce a wide variety of proteins, including those with disulfide bonds.

The classic genetic engineering approaches in prokaryotes often use phage-derived RecET and lambda red recombinase-based recombineering (Tong *et al.*, 2021). *Escherichia coli* is the most preferred microorganism to express heterologous proteins for therapeutic use, as around 30% of the approved therapeutic proteins are currently being produced using it as a host. Owing to its rapid growth, high yield of the product, cost-effectiveness, and easy scale-up process, *E. coli* is an expression host of choice in the biotechnology industry for large-scale production of proteins, particularly non-glycosylated proteins, for therapeutic use. The availability of various *E. coli* expression vectors and strains, relatively easy protein folding mechanisms, and bioprocess technologies, makes it very attractive for industrial application (Baeshen *et al.*, 2015). *E. coli* is a typical prokaryotic expression system and one of the most attractive heterologous protein producer. The expression of proteins in this system is the easiest, quickest and cheapest. To date reformed *E. coli* is the extensively used cellular host for foreign protein expression because of its rapid growth rate which is as short as 20-30 minutes (Amitha Reena Gomes, 2016), capacity for continuous fermentation and relatively low cost. There are many commercial and non-commercial expression vectors available with different N and C terminal tags and many different strains are being optimized for special applications (Amitha Reena Gomes, 2016).

There are also problems related to the use of *E. coli* as production host. These problems can be grouped into two categories: those that are due to the sequence of the gene of interest and those that are due to the limitations of *E. coli* as a host (Amitha Reena Gomes, 2016).

In the first category again there are three ways in which the nucleotide sequence might prevent efficient expression of a foreign gene. Firstly, the foreign gene may contain introns which would be a major problem since *E. coli* genes do not contain introns and so the bacterium does not possess necessary machinery for removing introns from transcripts. Secondly, the foreign gene might contain sequences which act as termination signals in *E. coli*. These sequences are perfectly harmless in the normal host cell, but in the bacterium it results in the premature termination and a loss of gene expression (Amitha Reena Gomes, 2016).

Besides this, the heterogenous expression may leads to the problem of disulfide bond formation, absence of chaperone and miss match codon. To rectify these problems mutated strains, co-production of chaperone and lowering the growth temperature have been used either separately or by combination (Costa *et al.*, 2014). With this background, In this experiment the recombinant chimeric cry 2Ax gene has been cloned with his and fusion tags, expressed in *E. coli* and toxicity assay was performed with *Helicoverpa armigera* and *Spodoptera litura*.

MATERIALS AND METHODS

The Department of Biotechnology, Centre for Plant Molecular Biology and Biotechnology, *Bacillus thuringiensis* lab, Tamil Nadu Agricultural University, Coimbatore, is where all the laboratory works has been carried out.

Reagents

We bought PCR purification kits and DNA isolation kits from Sigma (www.sigma-aldrich.com/genelutehp.) and Geni (<http://geneilabs.com>), respectively. T4 DNA ligase and restriction endonucleases were acquired from New England Biolabs (<http://www.neb>).

Plasmid DNA isolation, plasmid DNA purification and gel DNA extraction kits were purchased from Sigma. 2-log DNA ladder was procured from NEB. All other reagents were purchased from Sigma.

Bt, E. coli strains and PET 28a vector

A recombinant *Bt* (4Q7) transformed with pHT vector of Cry 2Ax gene was utilized as a donor in this experiment. Novogen, USA (<http://www.novogene.com>) supplied pET 28 a, *E. coli* strain DH5 α , and BL 21.

Media, growth condition

Bt and *E. coli* strains were cultured using Luria-Bertani (LB) broth (Bertani, 2004) in accordance with standard procedure. *E. coli* harboring PET 28a and its derivatives were mass cultured using kanamycin marker (100 μ g/ml) as directed by manufacturer.

DNA isolation

The genomic DNA of the recombinant *Bt* (4Q7) strain was extracted using Genei kit. (Na *et al.*, 2025). The quantity and quality of DNA was analyzed using the Agarose gel electrophoresis (Guijun Miao *et al.*, 2020) and NanoDrop spectrophotometer 2000.

Gene, Primers and Vector selection

The Cry 2Ax gene (1.9 Kb) of 4Q7 *Bacillus thuringiensis* DNA was used as a template. Gene-specific primers (as given below) were used for amplification. Two sets of primers were designed along with restriction sites on the basis of vector (pET 28) map Figure 1. Primer sets with ECoR1 and Hind III and other with NCO 1 and Hind III. The second set of primers (NCO 1 and Hind III) was designed not to cut the fusion tags, while the first set of primers (Eco R I and Hind III) was designed to cut the fusion tags during PCR. The primer sequence details are as follows.

S. no. Primer	Primer Sequence with restriction enzyme	Restriction enzyme
1 Forward	5' GCTCTAGATATTTAAGGAGGAATTTTATATGAA 3'	Eco R 1 (GCTCTAGA)
2 Forward	5'CCATGGGTAATGTATTGAATAGTGAA3'	NCo 1(CATGGGT)
3 Reverse	5' ACAAGCTTAGTTAATAAAGTGGTGAAGATTA 3'	Hind III (AAGCTT)

PCR (Cry 2Ax), gene purification

PCR was done in an Eppendorf thermal cycler. The PCR mixture (25 μ l) containing DNA (30 ng), PCR buffer (10X: 2.5 μ l), 75 μ M

each of dNTPs, 50 ng each of forward and reverse primers and 1.5 Units of *Taq* DNA polymerase have been used. The PCR was performed for 30 cycles at 60°C annealing

temperature and 2 minutes extension time. The PCR products were purified using ultraclean DNA purification kit (SIGMA USA, <https://www.sigmaaldrich.com>). The quantity and quality of amplified products was analyzed using the Agarose gel electrophoresis (Guijun Miao *et al.*, 2020) and NanoDrop spectrophotometer 2000.

Plasmid DNA (pET 28a)

pET 28a plasmid of *E. coli* (DH 5 α) strains was cultured in LB kanamycin broth at 37°C for overnight. Plasmid DNA was isolated using plasmid isolation kit (Sigma, USA) as per the instruction given by the manufacturer. Quantity and quality of DNA were analyzed through agarose gel electrophoresis (Guijun Miao *et al.*, 2020) and nanodrop reading (Thermo USA)

Restriction digestion (RD) of vector, gene

Two sets of RD were performed. Enzyme concentration was one unit for every μg of DNA. First RD set contains the restriction enzymes (Hind III and Eco R1), vector, insert, and buffer (BSA). The second RD set covers insert, vector, buffer (BSA), and restriction enzymes (Hind III and NCO 1). The restriction digestion was done in an Eppendorff tube at 4°C for one hour (Ramalakshmi *et al.*, 2022).

RD products clean up

Restriction digested products were purified by following the protocol provided by manufacturer Sigma Aldrich, Mumbai.

Ligation/cloning and Transformation

There were two sets of ligations performed. Cry 2Ax gene in one set, cry 2Ax gene with fusion tags in another. T4 DNA ligase (1 μl), buffer (2 x), vector (75 ng in 4 μl), insert (25 ng in 3 μl), and ligation water (10 μl) were combined and stored at 22°C for one hour (Tianyuan Su *et al.*, 2019).

The ligated mixtures were transformed into *E. coli* (DH 5 α) competent cells by heat shock method separately (Tianyuan Su *et al.*, 2019 and Ramalakshmi *et al.*, 2022).

Colony, Plasmid PCR and Restriction digestion (RD)

The transformed colonies of *E. coli* carrying the cry2Ax gene of pET 28 (a) were chosen based on the complementation test, and the colonies with self-ligated vector were removed

(Ramalakshmi *et al.*, 2022). For verification, the transformed *E. coli* colonies and their plasmid DNA were subjected to PCR using primers specific to the cry2Ax gene.

pET 28a plasmid of *E. coli* transformants (DH 5 α) were screened by plating the transformed colonies in LB kan (50 ppm) plate. Colony (Jamal Mahm, 2017), plasmid PCR and restriction digestion (Motohashi, 2019) were done to confirm the positive transformants. Gene specific primers as mentioned above were used for colony and plasmid PCR (Horecka, 2014). Confirmed colonies with positive clones were used for restriction digestion. Digestion of positive clone was done with two set of restriction enzyme as per two types of clones. Eco R1 and Hind III for Cry 2Ax gene, NCO 1 and Hind III for Cry 2Ax gene with fusion tags. Double digestion and single digestion were carried out. The results were confirmed by agarose gel electrophoresis.

Transfer of clone between *E. coli* strains

Positive clones (vector + insert) was isolated from recombinant DH 5 α strains and purified using Sigma Kit, USA. The purified clone/plasmid DNA was transferred into BL 21 (*E. coli*) expression strain. *E. coli* competent cells preparation and transformation were done by following standard protocol of Sambrook *et al.* (Ramalakshmi *et al.*, 2022 and Du *et al.*, 2021).

Expression of Cry 2Ax ORF of pET 28 (a) in *E. coli* (BL 21)

Cry 2Ax of pET 28a plasmid in BL 21 was cultured in LB broth (50 μg of kanamycin / ml) at 37°C overnight. BL 21 plain strain without plasmid was taken as a negative control. One percent mother culture of recombinant strains of two clones viz., Cry 2Ax with tag and without tag were inoculated into 25 ml LB broth under 37°C separately until to reach 0.6 OD at 600 nm. 1mM of IPTG (Isopropyl β -D-1-thiogalactopyranoside) was inoculated into the log phase culture for induction and kept under 30°C for 6 hrs. The broths were used for protein extraction by following the procedure of Ramalakshmi *et al.*, 2022.

SDS PAGE and Quantification of cry 2Ax protein

The gene expression/protein synthesis was analyzed by SDS-PAGE on separating gel of 9 percent w/vacrylamide (Nowakowski, 2014).

Proteins were quantified using known concentration of BSA as a standard. BSA Stock (10µg/µl) was diluted into 0.5, 1.0, 2.0 µg for SDS in such a way to calculate the concentration of recombinant protein after dilution. Dilutions were made based on protein expression in SDS PAGE after standardization. Since the Cry 2Ax protein with tags showed thick protein band, maximum dilutions viz., 1/7 and 1/10 and in case of Cry 2Ax protein without tags showed thin protein band 1/3, 1/4 and 1/5 dilutions were taken for comparison and quantification. Protein quantification was done by following the procedure described by Caballero *et al.* 2020.

Sequencing analysis

Positive clones of *E. coli* culture were sent for sequencing analysis. Homology search were conducted through Basic Local Alignment Search Tool (BLAST) at National Centre for Biotechnological Information (NCBI) (www.ncbi.nlm.nih.gov/Blast). The deduced amino acid sequence was generated by Bio Edit (Ramalakshmi *et al.*, 2022).

Bioassay for insecticidal activity

Recombinant proteins (Cry 2Ax alone and 2Ax with fusion tags) were tested for toxicity assay. *E.coli* (BL 21) plain strain protein was used as absolute negative control. Semi-synthetic diet (Patel *et al.* 1968) dispensed inside the 1.8 ml cryovial (Tarson®; 1 cm dia.) were inoculated with 7 days old larva of *Helicoverpa armigera* and *Spodoptera litura*. Recombinant proteins (10 µl) were spread on the surface of the vial and dry for 30 min. Ten vials were maintained for each treatment with three replications. Larval mortality was recorded periodically (1-7 days) (Saleem F, Shakoori AR 2017).

RESULTS

PCR (Cry 2Ax) followed by sequencing of recombinant Bt 4Q7

PCR results confirmed that positive amplification of Cry 2Ax gene (1.9 Kb) from genomic DNA. The amplification product showed bright bands at 58°C in 2% agarose gel (Figure 1). Cleanup product showed bright bands at 58°C in 2% agarose gel (Figure 2). The sequencing result confirmed that cry 2Ax gene homology under NCBI (BLAST).

(Annealing temp: 60°C, Extension time: 2 min)

Primers: 2IF2: TGAATTCATGAATAATGT
ATTGAATAGTGGAA (Eco R 1)

2IR₂a: ACAAGCCTTAGTTAATAAAGTGGT
GGAAGATTA (Hind III)

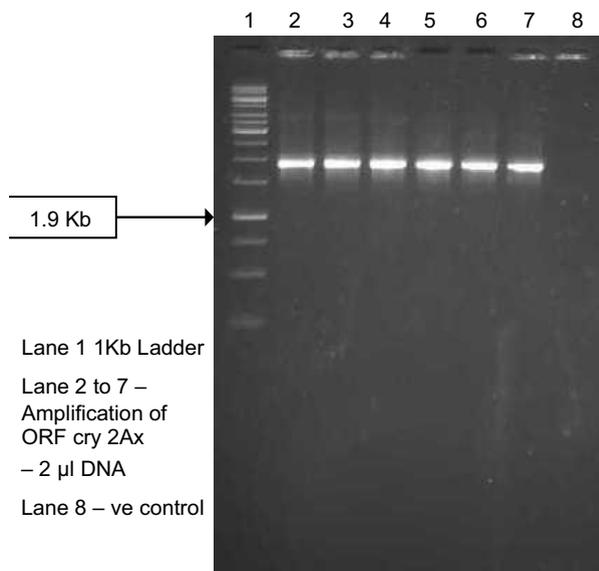


Figure 1: PCR amplification of the Bt 2Ax strain

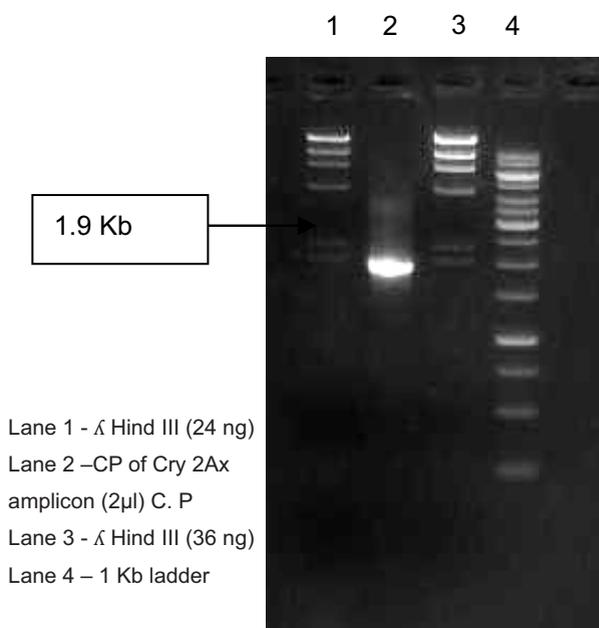


Figure 2: Column purification (CP) of PCR amplified products

Nanodrop: 242.0 ng/ul

Confirmation of Plasmid DNA

The concentration of plasmid DNA (100 ng/ul) was confirmed by gel electrophoresis using λ Hind III (Figure 3). The RD of single and

double digestion of vector also confirmed the pET 28a (+) vector (5.3 Kb) in gel electrophoresis (Figure 4).

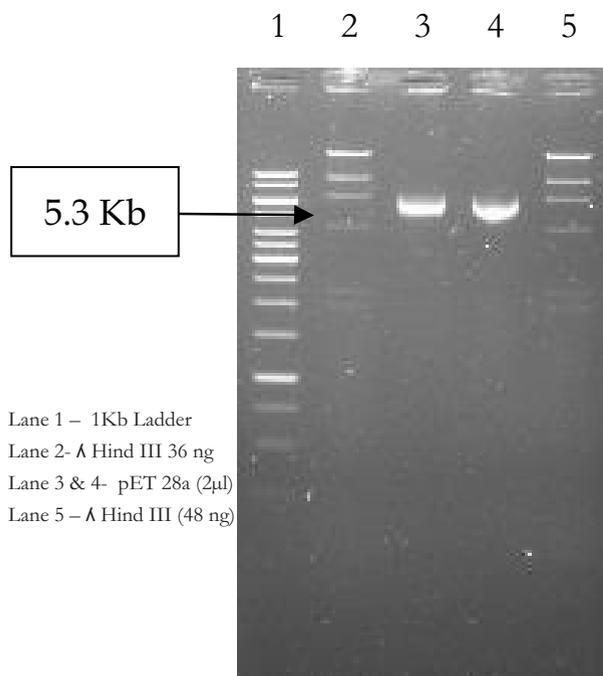


Figure 3: Concentration checking of isolated Plasmid DNA (pET 28a)

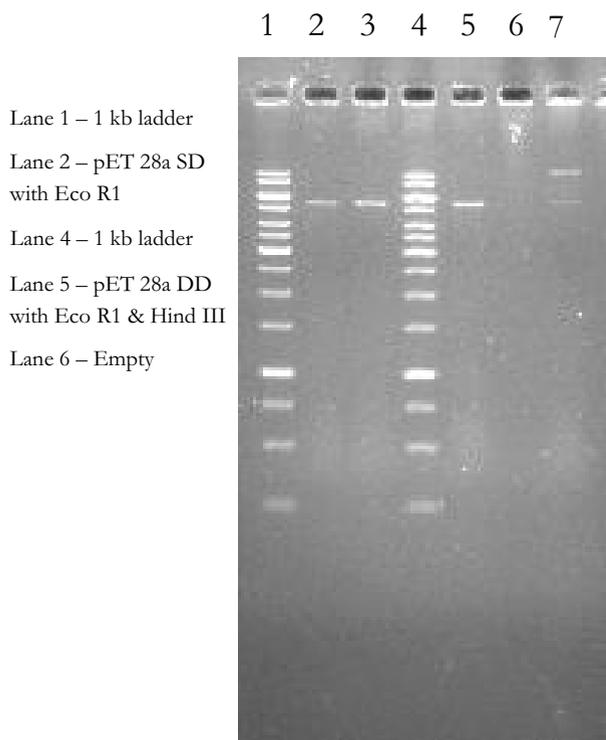


Figure 4: Confirmation of plasmid DNA through Restriction digestion

Concentration checking of Vector and insert after cleanup

Concentration of the Cleanup products viz., Vector (85 ng/ μl) and insert (135 ng/ μl) were confirmed by agarose gel electrophoresis (Figure 5)

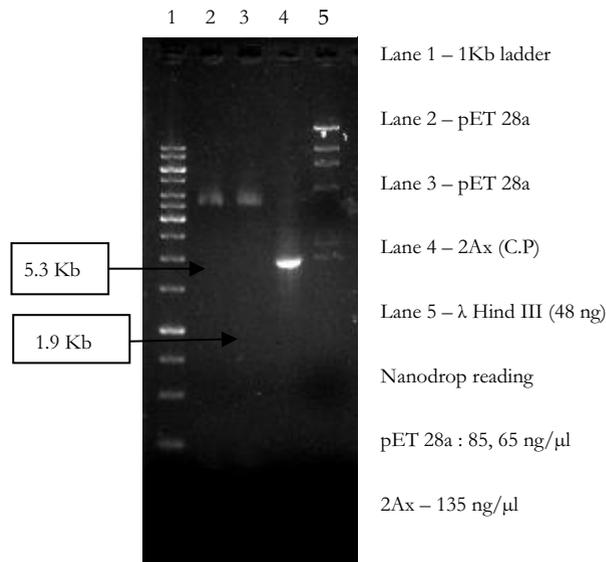


Figure 5: Concentration checking of vector and insert after

Confirmation of Ligation and transformation

White colour colonies of *E.coli* (DH 5 α) strain in Lb Kan plate confirmed the ligation. Blue colour colonies were confirmed as a non ligated one due to the expression Lac Z gene whereas the expression was masked by replacement of cry 2 Ax gene in white colonies.

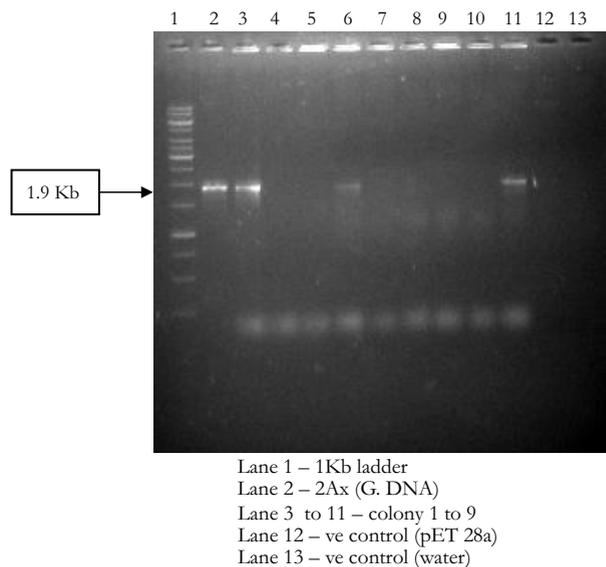


Figure 6: Colony PCR after transformation
Fragment size: 1.9 kb

Colony PCR, Plasmid PCR and Sequencing

Positive transformant containing clones were confirmed by colony PCR (Figure 6) with band size of 1.9 Kb (Cry 2Ax gene). The agarose gel electrophoresis result of Plasmid PCR also showed 1.9Kb band size, which confirmed that positive amplification of cry 2Ax gene from recombinant plasmid (Figure 7). PCR followed by the sequencing results also confirmed the 1.9 Kb size of cry 2Ax gene size.

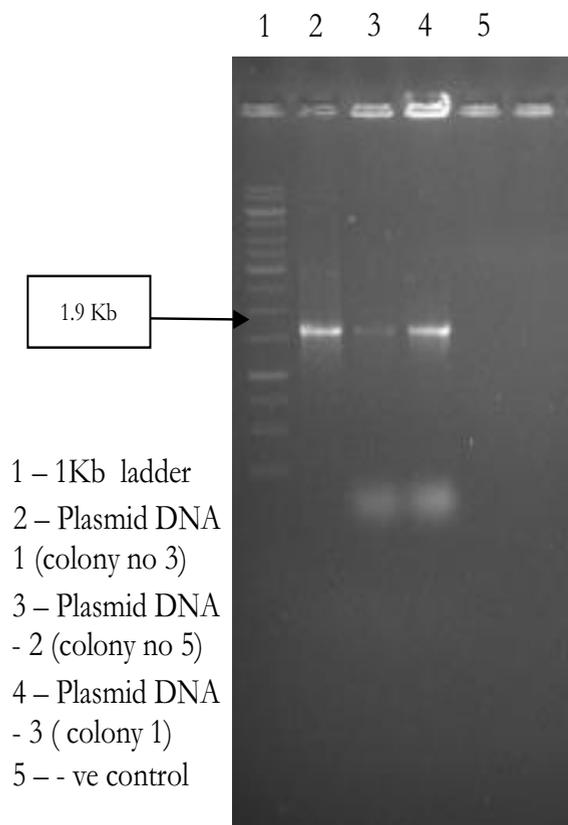
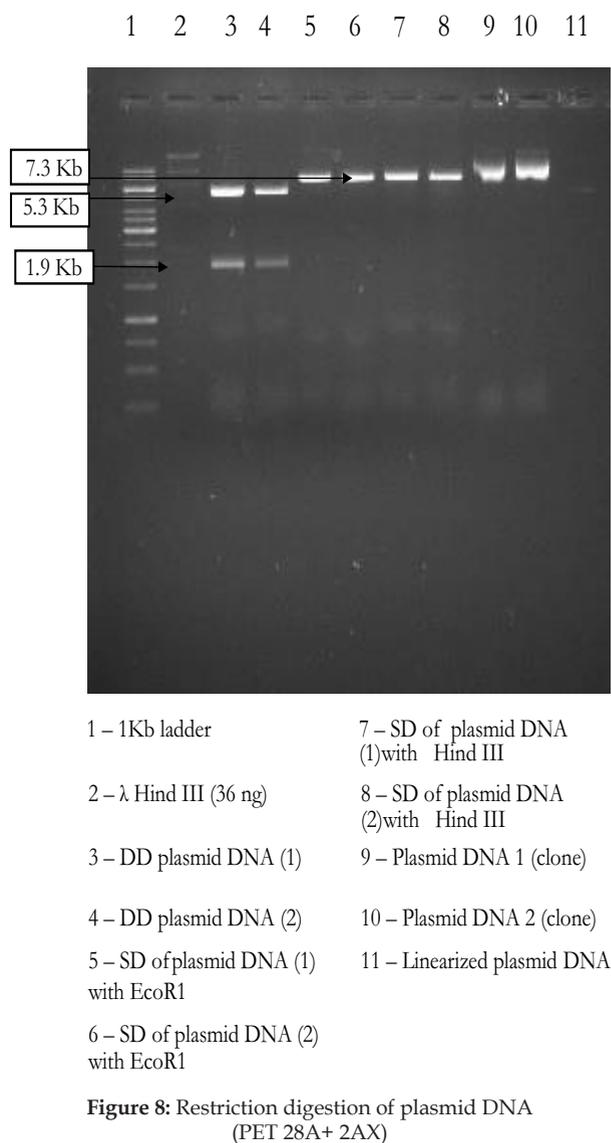


Figure 7: Plasmid PCR (pET 28a + 2ax)

RD of cry 2Ax in pET 28a

Ligation of the recombinant plasmid with insert (Cry 2Ax) was also confirmed by the restriction digestion. The band size in agarose gel electrophoresis confirmed the vector and insert size (Figure 8). The recombinant plasmid underwent double digestion, yielding two bands measuring 5.3 Kb and 1.9 Kb. where the insert region was indicated by 1.9 Kb and the vector size was indicated by 5.4 Kb. A single digestion resulted in a single band that was 7.2 Kb in size, including the insert and vector. The outcomes amply demonstrated that the cry2Ax gene was significantly ligated within the vector region.



- | | |
|--------------------------------------|--|
| 1 – 1Kb ladder | 7 – SD of plasmid DNA (1)with Hind III |
| 2 – λ Hind III (36 ng) | 8 – SD of plasmid DNA (2)with Hind III |
| 3 – DD plasmid DNA (1) | 9 – Plasmid DNA 1 (clone) |
| 4 – DD plasmid DNA (2) | 10 – Plasmid DNA 2 (clone) |
| 5 – SD of plasmid DNA (1) with EcoR1 | 11 – Linearized plasmid DNA with EcoR1 |
| 6 – SD of plasmid DNA (2) with EcoR1 | |

Figure 8: Restriction digestion of plasmid DNA (PET 28A+ 2AX)

Transfer of clone into *E. coli* expression strain (BL 21)

The growth of colonies in LB kan plate confirmed that the successful transfer of clone (pET 28 (a) + Cry 2Ax) from *E.coli* DH5 α to BL 21 strains.

Protein expression followed by quantification (Schlicke and Brakmann, 2005)

The SDS page result was 65 kDa, which was equivalent to the Bt protein cry 2Ax (Figure 10). The clone with tag had 2.0µg/µl of protein, while the clone without tag had 0.8µg/µl. It was evidenced that the recombinant *E. coli* strain (Figure 10) had substantially higher protein levels than Bt (0.4 µg/µl).

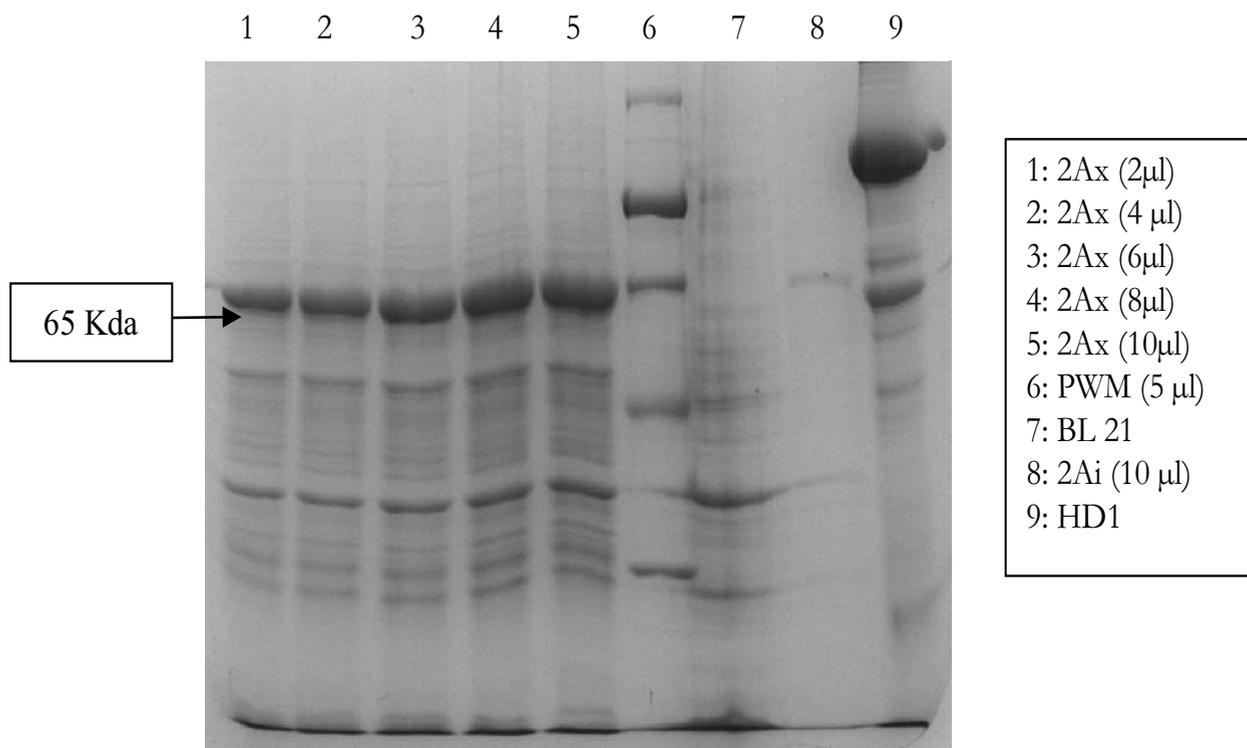


Figure 9: Protein harvesting through induction from BL 21 transformed with pET 28a + Cry 2Ax

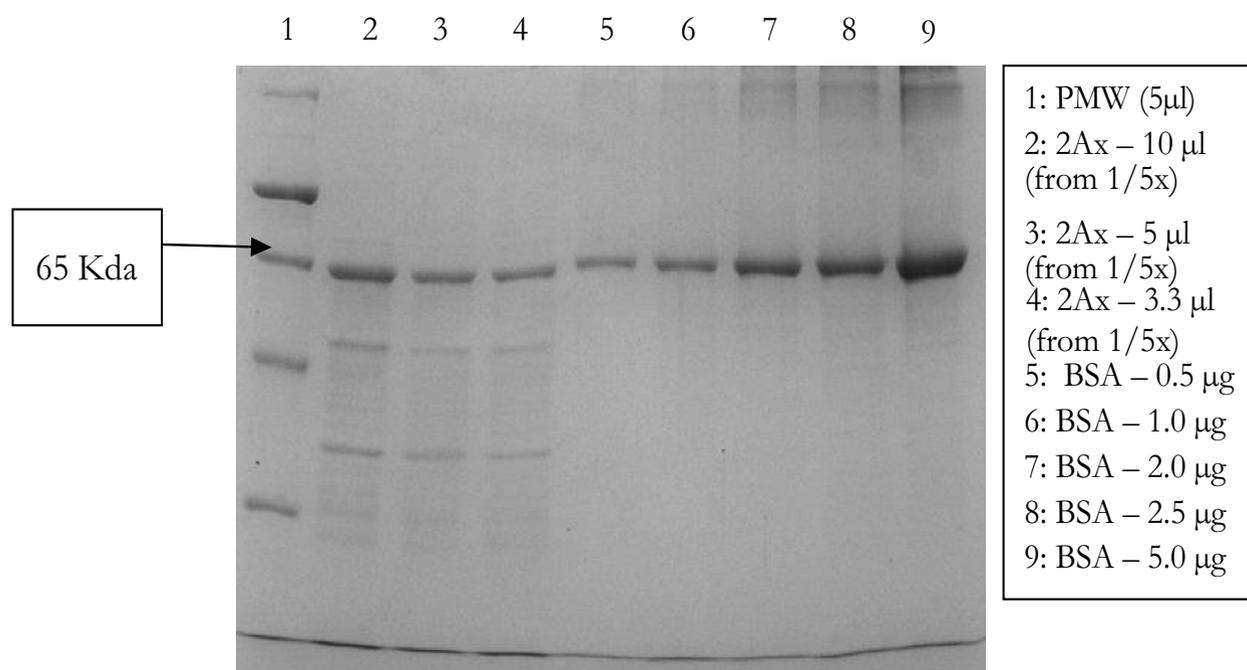


Figure 10: Quantification of 2Ax protein using BSA standard

Bioassay

The bioassay results for cry 2Ax protein without tag and cry 2Ax protein with tag showed 100% and 70% mortality, respectively

(Table 1). The bioassay result's showed that there were notable differences between the recombinant proteins activity with and without tags.

Table 1: Bioassay of cry 2Ax protein against pink boll worm (*Spodoptera litura*)

Treatment	1 Day	2 Day	3 Day	4 Day	5 Day	6 Day	7 Day
Control	0	0	0	0	0	20 % 1 missing	20 % 1 missing
HD 1	10%	80%	100%	100%	100%	100%	100%
Cry 2Ax	0 %	30%	90%	100%	100%	100%	100%
Cry 2Ax with tags	0 %	30%	50%	50%	50%	60%	70%



Figure 11.1: Bioassay of pink pigmented boll worm (*Spodoptera litura*) without any protein (Control)



Figure 11.2: Bioassay of pink pigmented boll worm (*Spodoptera litura*) with Cry 2Ax alone (Heterologus *E.coli*)

DISCUSSION

Gene transfer from genome to genome is being leading practice in current science for the human welfare. Biopesticide development from recombinant cry gene was prolonged follow-up practice in biotechnological science to study gene cloning for the past several decades. Even though several decades' research had been undergone regarding heterogenous gene expression, there is a lack of study about the function of native chaperones. Apart from that spores (or) cry protein are expressed based on stress condition (or) whenever the signal molecules initiated. This may cause variation in the expression of recombinant cry gene. Cry gene expression was controlled by cry gene promoters and non

cry gene promoters. The role of these cry gene and non cry gene promoter was mediated by stress depended and induced mechanism of the native strain which was a question mark in case of recombinant cry protein expression in *E.coli* strains.

All the human, animal and microbial cell depends on the chaperone mediated network to form successful protein transfer, placement and expression and their by preventing misfolded protein formation (Balchin *et al.*, 2016). Some chaperones use the ATP binding hydrolysis and some do not use for chaperone activity. In general ATP independent chaperone don't interact with native chaperone instead of that bind with partially folded client protein and try to avoid protein aggregation

(Suss and Reichmann, 2015 and Burmann and Hiller, 2015). Some exception like Spy is the ATP independent chaperone was incapable for facilitating their refolding also observed (Burmann and Hiller, 2015 and Suss and Reichmann, 2015).

Wu *et al.* (2019) reported that too tight binding inhibit protein folding and too loose binding favour protein aggregation. Cry gene transcriptional regulation has been divided into sporulation-dependent promoters and sporulation independent promoters. Sporulation depended promoters are classified into sporulation-specific sigma factors SigK and/or SigE. SigA factor controls the sporulation-independent promoters. Accessory factors also contribute to the transcriptional regulation of cry gene expression such as Spo0A, ORF2, and CcpA (Peng *et al.*, 2018).

E. coli expression systems are commonly used for the expression of cry genes. Tac promoters and T₇ are commonly used to for the expression of cry genes carried on pET or pGEX series vectors with His or GST tags (Qi peng *et al.*, 2019).

Even though, some cases *E. coli* fails to express eukaryotic genes due to the lack of disulphide bond formation, post translational modifications and fails to form codon. This may be due to the variation in the codon sequence of Eukaryotes and Prokaryotes. Fast expression of recombinant protein leads to formation of insoluble aggregates due to the failure of chaperone mediated network to prevent misfolded protein formation (Ajmal, 2023).

The experiential results revealed positive cloning of cry 2Ax gene in pET 28(a). There was a variation in the transgenic expression of cry 2Ax protein with and without tags. Experimental result revealed protein expression was higher (2.0 µg / µl) from transformed colonies with tag than without tags (0.8 µg / µl). Gene tagging help for expression but somehow affect the cofactor or chaperone binding into the fresh or newly released protein (Köppl *et al.*, 2022). Hence more proteins were accumulated in the cells. Cells rely on a comprehensive chaperone network to mediate protein folding and prevent protein aggregation (Balchin *et al.*, 2016). The chaperone specific to the native gene was missed in the case of chimeric cry 2Ax gene in recombinant *E. coli* DH 5 α strain.

Hence there was more protein accumulation in the SDS page of recombinant tagged gene and the proper folding and release in appropriate place was absent, this will cause insoluble and non functional protein

There was low quantity of other extracellular protein along with 2Ax protein with fusion tags but high amount of extracellular protein were observed in case of 2Ax protein without tags. Tagging the proteins with tags during recombination increases protein aggregation and decreases the protein folding, toxicity and solubility. Cry 2Ax without fusion tags and expression in *E. coli* not disturb the protein folding and toxicity. The bioassay results confirmed that the cry 2Ax protein (without tags) results showed 100% mortality where as cry 2Ax protein (with tags) showed 80% mortality.

CONCLUSION

Complete expression of foreign genes in *E. coli* is dependent on a number of factors. If the heterogenous protein expression was perfect then scale up process will start. However efficient expression of heterogenous gene and scale up process depends similarity between donor and recipient genome. This experimental result concluded that his and fusion tags in pET 28a(+) vector decreases the positive way of protein folding, transport and release of cry 2Ax gene in *E. coli*.

Abbreviation: *Bt*: *Bacillus thuringiensis*, SD: Single digestion, DD: Double digestion

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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REVIEW ARTICLE

Pteridophytes as Natural Scavengers of Soil Arsenic: From Physiological Foundation to Environmental Maneuvering

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ABSTRACT

Arsenic (As) contamination in soil poses a serious threat to ecosystem and human health. Different physical and chemical methods are employed to mitigate this problem, but they have several inherent drawbacks including high cost, energy intensive and eco-invasive nature. Whereas phytoremediation is an eco-friendly, cost-effective and sustainable strategy for remediation of soil As contamination. Pteridophytes have evolutionary advantages that allow them to accumulate contaminants from soil, thus acting as natural scavenger of soil contaminants. Chinese brake fern (*Pteris vittata* L.) is credited with the distinction of being 'first' arsenic hyperaccumulator exhibiting remarkable potential for arsenic removal from contaminated soils up to 22,630 mg kg⁻¹ As in its dry mass. In this review starting with a brief outline of the taxonomy of phytoremediation, the molecular mechanisms of accumulation, translocation, and detoxification of As in pteridophytes have been highlighted with special reference to *P. vittata*. Further, the means and measures of maneuvering phytoremediation have been discussed with an objective of enhancement of its performance and success in environmental remediation.

KEYWORDS

• Arsenic • Bioaccumulation • Hyperaccumulators • Phytoremediation • *Pteris vittata* • Soil

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INTRODUCTION

Arsenic (As) is a redox active toxic metalloid classified as Class I category of carcinogen the International Agency for Research on Cancer (IARC, 1987)(Y. Li *et al.*, 2024). Based on abundance, As has been ranked at 20th position among the elements available in earth's crust. Pollution of As in soil and water has become a burning environmental issue in many parts of the world, especially in South and Southeast Asia. More than 60 million people are estimated to be at risk of As poisoning in Bengal delta comprising major part of West Bengal, India and Bangladesh as its hotspot (Mondal *et al.*, 2025). The sources of As contamination in soil and water can be natural (geological and biological) as well as anthropogenic. Currently, unplanned development, industrialization and resource mismanagement have added fuel to the fire of the pre-existing As contamination. In such areas, even a single hand pump exposes a large section of the population to As-contaminated drinking water and its toxicity. Main staple food of these regions is rice, cultivated twice or thrice a year, relying on rainwater as well as heavily on irrigation. Therefore, significant amount of As get deposited gradually in agricultural soil, and enters easily into the food chain. Rice and other crops grown in this soil is likely to be contaminated with As, while its prolong consumption poses pernicious human health risks and diseases such as peripheral neuropathy, cardiovascular diseases, miscarriage, prostate and skin cancer (Y. Li *et al.*, 2024).

Conventionally, different physical and chemical approaches are adopted to remove As from soil but most of them are expensive, labour-intensive, and eco-invasive in nature, which can alter soil physicochemical properties, and adversely affect the beneficial soil microbiome (Jassal *et al.*, 2025; F. J. Zhao *et al.*, 2022). Contrarily, phytoremediation offers a green, eco-friendly, sustainable, and cost-effective alternative demanding low or no external inputs but by involving (periodic growing and harvesting) plants capable of accumulating As in their body parts (Capobianco *et al.*, 2026; Huslina *et al.*, 2024). In recent years researchers have focused on employing plants to cleanup and restore arsenic-contaminated soils. Non-edible plants endowed with high growth rate and contaminant accumulation potential

are ideal candidates for remediating arsenic-contaminated soils safely and effectively. Among different plant groups vascular cryptogams (belonging to Pteridophyta) appear as the best choice as potent soil As phytoremediation agents. Arsenic hyper-accumulating pteridophytes (ferns) belong to order Pteridales. Well known As hyper-accumulating ferns are *Pteris vittata* and *Pityrogramma calomelanos*, which can accumulate up to 19,300 mg kg⁻¹ and 11,600 mg kg⁻¹ As respectively, in respective dry mass after 78 days from soils contaminated with As (Yong *et al.*, 2010). Being an inherently slow process a successful and substantial remediation outcome demands phytoremediation to become a part of long-term remediation strategy. Such disadvantages of phytoremediation can be overcome through adoption of a holistic approach known as phytomanagement (Pandey & Bajpai, 2019). The main objectives of phytomanagement is ecosystem rehabilitation with substantial land use. Phytoremediation efficiency of pteridophytes can be improved by applying chelating agents, organic amendments, beneficial bacteria, genetic engineering and omics approaches (Parida *et al.*, 2024). Nano-phytoremediation is relatively recent phytoremediation approach which combines the use of nanoparticles in phytoremediation technology, but its application is till now limited due to lack of inadequate research evidences (Prakash & Chandran, 2023; Usman *et al.*, 2025).

TAXONOMY OF PHYTOREMEDIATION

The phytoremediation process refers to using plants, along with their associated soil microorganisms, to minimize the concentration, mitigate toxicant's stress and/or alleviation of toxicity of environmental contaminants. The term phytoremediation is derived from two words: the Greek word phyto, meaning "plant," and the Latin word remedium, meaning "to remove or remediate". Phytoremediation is an eco-friendly, efficient, cost-effective and solar-driven technique which can be used efficiently for remediation of inorganic (viz. heavy metals, metalloids, radionucleotides etc.) and organic pollutants [viz. polychlorinated biphenyls (PCB), polynuclear aromatic hydrocarbons (PAH), Hexachlorocyclohexane (HCH), Dichlorodiphenyltrichloroethane (DDT),

Polybrominated diphenyl ethers (PBDE), Chlorpyrifos (CPF) etc.]. Hyperaccumulator plants can be grown on toxic heavy metals contaminated sites to remedy the polluted soil. Phytoremediation has earned popularity as nature-based solution (NbS) because of its innocuous nature and affordability arising from lower maintenance and installation costs compared to conventional methods (Bhat *et al.*, 2025). Thus common people termed phytoremediation as “green clean” (Pham *et al.*, 2022).

DIFFERENT PROCESSES OF PHYTOREMEDIATION

The processes involved in phytoremediation are phytoextraction/phytoaccumulation, phytodegradation, phytofiltration, phytostabilization, and phytovolatilization.

1. Phytoextraction

It is also known as phytosequestration or phytoaccumulation or phytoabsorption. In this uptake process contaminants are taken up from the environmental medium by plant roots and subsequently translocated and accumulated in above-ground plant biomass. The efficiency of contaminants accumulation in above-ground biomass of the plants, especially in shoot depends on their ability to take up that particular contaminants with high efficiency and subsequent translocation to their shoots avoiding complexation in their roots. This contaminants translocation is very crucial for the effective phytoextraction as the root biomass is not generally efficient (X. Cao *et al.*, 2025; Islam *et al.*, 2024).

2. Phytofiltration

It is also known as rhizofiltration (by plant roots) or caulofiltration (by excised shoots) or blastofiltration (by seedlings). This process is associated with contaminants removal from waste waters or surface waters by plant root system. Actually phytofiltration reduces metals and metalloids mobility in sediment by eliminating pollutants from aqueous environment. Here the movement of contaminants to underground water is minimized due to their adsorption or absorption by plant roots (Bokhari *et al.*, 2022).

3. Phytodegradation

The process involves the degradation of only organic pollutants in medium by plant

enzymes such as dehalogenase and oxygenase (Praveen and Pandey, 2020). It is also known as phytotransformation. Several organic xenobiotic compounds are accumulated in plant bodies and detoxified through their metabolic activities. Phytodegradation is independent of rhizospheric microorganisms. Due to the non-biodegradable nature of heavy metals and metalloids, phytodegradation is applicable for the removal of organic pollutants only (H. Liu *et al.*, 2024). Although toxic metal(loid)s may undergo biotransformation process.

4. Phytovolatilization

In phytovolatilization process plants absorb metals and metalloids from the soil, transform them into less toxic, volatile, or organic forms, and subsequently release the transformed compounds into the atmosphere. Phytovolatilization is only feasible for organic pollutants and certain metals and metalloids that have volatile species like Hg, Se, As. Phytovolatilization is a controversial phytoremediation technique as it releases the volatile toxic pollutants from one segment (soil) to atmosphere for further dispersion through air from where it may be redeposited, just shifting the pollution burden from one environmental medium to another (Shen *et al.*, 2022).

5. Phytostabilization

It is also known as phytoimmobilization. Phytostabilization refers to the stabilization of contaminants in contaminated soil by using certain plants, thereby, immobilizing contaminants in the medium. This technique is very much useful to minimize the bioavailability and mobility of contaminants in the environment. It also decreases the rate of metal(loid)s leaching into the ground water. The immobilization of contaminants by plants occurs through various mechanisms such as sorption onto root surfaces, complexation, precipitation, and valence reduction of metals or metalloids within the rhizosphere (Siunova *et al.*, 2025). However, phytostabilization is considered a temporary mitigatory approach because the contaminants still remain in the soil with their restricted mobility. Phytostabilization indeed is a management strategy for stabilizing the potentially toxic elements (Borbón-Palomares *et al.*, 2024).

6. Phytodesalination

Phytodesalination is associated with salts removal from saline soil by using halophytic

plants to ensure normal plant growth and development. Naturally halophytic plants are adapted better to salt stress conditions than salt-sensitive glycophytic plants to cope with heavy metal(loid)s in the soil (Manousaki & Kalogerakis, 2011).

7. Rhizodegradation

This technique is associated with rhizospheric microorganisms mediated organic contaminants breakdown in the soils. Plants release exudates rich in nutrients such as simple carbohydrates, amino acids, flavonoids etc., in the rhizosphere helps to increase metabolic activities of microbes that enhance the degradation of organic pollutants in the rhizosphere. Apart from these plant roots also secrete certain exoenzymes which is capable of degradation of organic contaminants in soils (K. M. Yang *et al.*, 2023).

EFFICIENCY OF PHYTOREMEDIATION

The main and most useful technique of phytoremediation is phytoextraction to remove the contaminants from polluted soils and water (Praveen & Pandey, 2020).

$$\text{Bioconcentration factor (BCF)} = \frac{\text{Concentration of the target metal(loid) in the harvested plant part(tissue)}}{\text{Concentration of the same metal(loid) in the soil}}$$

2. Translocation factor (TF)

The efficiency of a plant species in translocation of accumulated metal(loid) from

$$\text{Translocation factor (TF)} = \frac{\text{Concentration of the target metal(loid) in plant shoot}}{\text{Concentration of the target metal(loid) in plant root}}$$

ARSENIC

Arsenic is considered as one of the most enigmatic elements known to humankind. It is believed that Albertus Magnus, a German scholar and alchemist of the 13th century, was the first to discover and isolate elemental As from its mineral sources (Paul *et al.*, 2022). Arsenic is ranked as the 53rd most abundant element in the Earth's crust. Inorganic arsenic continues to hold the highest position in the priority list of hazardous contaminants as enumerated by the Agency for Toxic Substances and Disease Registry (ATSDR, 2023). Arsenic is a metalloid having an atomic number of 33 and an atomic mass of 74.92 g mol⁻¹. Arsenic belongs to pnictogen group i.e. under group

Different factors like bioavailability of the contaminants in soils, properties of soils, redox state of the contaminants, concern plant species etc. regulate the efficiency of the phytoextraction. The screening and selection of hyperaccumulators for the phytoextraction of target metal(loid)s solely depend on the values of bioconcentration factor (BCF) and translocation factor (TF) (Huang *et al.*, 2025), BCF bears much significance in assessing the potential of a plant species for phytoextraction rather than the bulk quantum of metal(loid) concentration in shoot. The value of TF more than 1 refers the efficient translocation of accumulated metal(loid) from root to above ground parts (Cakaj *et al.*, 2024). Plant species with both BCF and TF being greater than one (>1) should be ideal for phytoextraction (Sharma *et al.*, 2023). Sometimes hyperaccumulators have BCF even up to 50 – 100 (Cluis, 2004).

1. Bioconcentration factor (BCF)

The efficiency of a plant species for a metal(loid) accumulation into its biomass from the surrounding environment is particularly determined by bioconcentration factor (BCF). It is calculated as follows (Gupta *et al.*, 2022).

roots to shoots is determined by translocation factor (TF), which is calculated as stated below (Gupta *et al.*, 2022).

15 and period 4 of the modern periodic table. The average As concentration in the earth's upper continental crust approximately 4.8 µg g⁻¹ (Schlesinger *et al.*, 2022). However, in many contaminated areas As concentration exceeds 300 µg g⁻¹ (Michopoulos, 2021). In the environment, As exists in four major oxidation states: -3, 0, +3, and +5, which vary according to the prevailing redox conditions and pH. Under oxic and generally alkaline groundwater conditions, arsenate [As(V)] remains as the dominant species whereas, arsenate [As(V)] gets reduced to arsenite [As(III)] under reducing redox condition of suboxic groundwater within deltaic aquifers. As(V) is 60 times less harmful than As (III)

(Mukherjee *et al.*, 2024). Under both oxidized and reduced conditions within the normal pH range (6.5-8.5) As mobilization in soil can take place (Q. Y. Chen & Costa, 2021; Rae, 2020). Arsenic exhibits chemical similarities with N and P because both belong to the same group 15 in the periodic table.

Methylated and organic As species represent key intermediates in the biogeochemical cycling of As. Biomethylation of inorganic As mediated by plants and microbes results in the formation of methylated As species such as monomethylarsonic acid (MMA) and dimethylarsonic acid (DMA) etc. Different organic As compounds including arsenobetaine, arsenosugars, and arsenolipids are found in marine organisms, and originate through complex biosynthetic processes. Inorganic forms of As are reported to be about 100 times more toxic than organic As compound (Mukherjee *et al.*, 2024). Although organic As species are more mobile than inorganic arsenicals, governing As transport and bioavailability in soil-water systems.

1. Sources of arsenic in environment

Arsenic is naturally found in the air, rocks, soil, sediment, water, plants, animals, and sediments, released into the environments through different process such as volcanic eruptions, weathering of rocks, erosion, and forest fires etc. Arsenic occurs naturally in more than 200 types of minerals, with nearly 99% of the total As present in rocks and minerals (Q. Y. Chen & Costa, 2021). Sulfidic minerals are the main source of As, predominantly exists in anaerobic conditions. However, As is associated with several rock-forming minerals, including phosphates, silicates, sulfides, and oxides. Apart from this arsenolite (As_2O_3), cobaltite ($CoAsS$), orpiment (As_2S_3), and olivenite (Cu_2OHAsO_4) are the important As containing minerals. Arsenic can be released into ground water through the dissolution of As-bearing minerals (Polizzotto *et al.*, 2006). Arsenic may also be released from river sediments to ground water under reducing redox condition. The As mobilization from iron oxides located in aquifer sediments has been reported to major causes of groundwater contamination. The sources of As due to anthropogenic activities are mining; burning fossil fuels; application of arsenical pesticides, fungicides; and also pharmaceutical drugs (Nriagu *et al.*, 2007).

2. Global status of As contamination

Both organic and inorganic form of As become a major public health concern, affecting hundreds of millions of people worldwide. The World Health Organization (WHO) and the Environmental Protection Agency (EPA) have limited maximum As concentration in drinking water $10\mu g L^{-1}$ while, As concentrations even below $40\mu g L^{-1}$ can pose serious health risks to humans (Mukherjee *et al.*, 2024). Globally, the highest concentrations of arsenic have been reported in metalliferous mining areas. As level reaches up to $2500 mg kg^{-1}$ as recorded in estuarine sediments in southwest England and $484 mg kg^{-1}$ in stream sediments from the Suratá river in Colombia (Ozturk *et al.*, 2021; Patel *et al.*, 2023). Both surface and ground water are contaminated with As in several countries of African subcontinent. Arsenic concentration reaches up to $6150\mu g L^{-1}$ in Rift valley of Ethiopia and Johannesburg in South Africa (B. K. Thakur *et al.*, 2021). Similarly, in North America and Latin America, several countries including Brazil, Argentina, Bolivia, Colombia, Chile, Ecuador, Cuba, Guatemala, Honduras, Peru, Nicaragua, Uruguay and Mexico have been reported to be affected by As contaminated ground water. Western part of the USA and many European countries including Turkey, Spain, Siberia, Romania, Hungary, and Greece also suffer from As contaminated ground water which has been reported to range between 30 and $4500\mu g L^{-1}$ (Q. Y. Chen & Costa, 2021; Patel *et al.*, 2023). In this count Bangladesh stands as a remarkable country, where 77 million people are exposed to mass As poisoning via drinking of As-contaminated ground water (Rehman *et al.*, 2021). Highest As concentration reported in ground water of Bangladesh is $4,730\mu g L^{-1}$ (Chowdhury, 2023). In India, occurrence of As in groundwater contaminated region is mainly distributed across two distinct zones: (i) the hard-rock regions of Karnataka and Chhattisgarh; (ii) alluvial region of Punjab, Uttar Pradesh, Bihar, Jharkhand, Manipur, Assam, and West Bengal (Chakraborti *et al.*, 2017). The surface soil of Korba basin area and Ambagarh Tehsil of Chhattisgar state have recorded exceptionally high levels of As up to $164 mg kg^{-1}$ and $4600 mg kg^{-1}$ respectively (Patel *et al.*, 2023). Ground water contamination by As in West Bengal was first reported in 1978, and now it has become one of the severely affected areas in the world (Chakraborti *et al.*, 2017). According

to Central Ground Water Board (CGWB), As contamination in groundwater was detected in nine out of the twenty-three districts of West Bengal, approximately 7 million people are suffering of arsenicosis having chronic symptoms (Chakraborti *et al.*, 2017). Nadia is the most affected district in West Bengal, with highest levels of As contamination in ground water. Besides, several districts in West Bengal, namely Malda, Murshidabad, North 24 Parganas, South 24 Parganas, Hooghly, Purba Bardhaman, Howrah and Kolkata have been reported to have As concentration in groundwater exceeding the WHO recommended permissible limit ($10 \mu\text{g L}^{-1}$) (Chakraborti *et al.*, 2017). The highest As concentration ($3,700 \mu\text{g L}^{-1}$) in groundwater has been recorded from a tubewell water sample in West Bengal, which is approximately 370 times higher than the WHO's recommended value of $10 \mu\text{g L}^{-1}$ (Chakraborti *et al.*, 2017).

3. Toxic effects of As on human

Arsenic is a non-essential toxic metalloid causing deleterious effects on every organ of human body as a result of interference in normal metabolic processes (Q.Y. Chen & Costa, 2021). Arsenic poisoning may be acute and chronic. Acute toxicity of As results in vomiting, nausea, severe diarrhoea; whereas prolonged exposure (5 - 10 years or more) causes chronic toxicity of As resulting in multisystem diseases with typical arsenical symptoms such as black foot disease, melanosis, and keratosis. According to National Research Council [NRC (1999)], in many As affected regions of India, children under 11 years have no arsenical symptoms though their biological samples exhibit high As levels. However, characteristic As induced skin lesion was manifested among children chronically exposed through drinking of higher As contaminated water ($\geq 1000 \mu\text{g L}^{-1}$) while nutritionally poor subjects showed such typical symptoms upon chronic exposure to relatively lower contamination level of As ($\geq 500 \mu\text{g L}^{-1}$) in drinking water (Biswas *et al.*, 2020; Rahman *et al.*, 2001). Arsenic tends to accumulate predominantly in the liver, kidneys, and muscles. Arsenic also a group 1 carcinogen promotes cancers of the lung, skin, kidneys, bladder, and prostate (NRC, 1999). Arsenic is a well-recognized human carcinogen, and no specific treatment exists for chronic arsenic poisoning, so effective management strategies primarily involve

minimizing exposure to As by controlling it at its source (Moulick *et al.*, 2021).

4. Effects of As on plants

Arsenic is toxic to plants depending on the types, nature and environmental factors. Plants show differential phenotypic features subject to dose and time of exposure of As. Chronic exposure of plants to As initially results in symptomatic whitish appearance, which subsequently leads to interveinal necrosis. It causes several detrimental effects on crops such as rice, wheat, maize, barley, and vegetables. Arsenic shows accumulation and biomagnification in human population along the food chain via the consumption of crops (Garg & Singla, 2011; Upadhyay *et al.*, 2019). Arsenic alters various metabolic pathways through inactivation of enzymes or competitive inhibition of phosphate (Pi). Excess generation of reactive oxygen species (ROS) and oxidative burst stand as key biomarker for As induced stress response in plant. The As stress is defended naturally by plants as reflected in higher activities of the antioxidative enzymes or mitigation through exogenous administration of amino acid proline. Apart from this, plants operate several adaptive mechanisms to cope with As toxicity such as up regulation of antioxidative defense system, hyperaccumulation, phytochelation, and reductive transformation [As(V) to As(III)], and subsequent sequestration in cell vacuoles (Biswas *et al.*, 2024; Patel *et al.*, 2023). Besides arsenate reductase enzymes, As(V) can also be reduced to As(III) by certain other enzymes such as glyceraldehyde-3-phosphate dehydrogenase and mitochondrial F1F0-ATP synthase. Furthermore, in place of inorganic phosphate, these enzymes may incorporate As(V) into biological molecules, leading to the formation of arseno-esters (Bakhat *et al.*, 2017). High As levels in soil or water damage vital biomolecules, inhibit biosynthesis of chlorophyll and antenna pigments, disrupt photosystems I and II, light harvesting complex II, and interfere Calvin cycle enzymes, thereby reducing photochemical efficiency and photosynthetic rate, which are key indicators of As stress in plants (Zulfiqar & Ashraf, 2022). Arsenic toxicity in plants reduces germination and respiration rate, decreases biomass and yield, reduces plant growth, accelerates senescence of leaves and sometime apoptosis under lethal dose (Bali & Sidhu, 2021).

PTERIDOPHYTES AS A POTENT PHYTOREMIATOR

Pteridophyta is an independent plant division under kingdom plantae, originated in earth approximately 400 million years ago. Pteridophytes are cosmopolitan in distribution, and are found in diverse habitat ranges, including rocky crevices, moist forest, shaded terrestrial or aquatic environments (Qian et al., 2022). Habit of pteridophytes ranges from small herb to long tree ferns. Pteridophytes are often known as vascular cryptogams due its well-developed vascular system and absence of seeds. Vascular tissues are devoid of vessels. They can serve as bioindicators of environmental contamination and ecological stability of a particular region. Because of their unique potential of uptake and accumulation of metals and metalloids certain pteridophytes can be effectively used as phytoremediator although they are not used as food or fodder. They exhibit the following characteristic traits and adaptive strategies that help them to remove toxic soil contaminants like As, as an effective phytoremediation agent: (a) Short life cycle that allows repeated harvesting and replanting, ensuring faster, continuous removal of soil As compared to plants with long life cycle; (b) Faster multiplication rate and biomass accumulation in shorter time period that accelerates As removal from soil involving more tissue to absorb and store As; (c) low maintenance because of thriving naturally without the need for extra care or maintenance; (d) Self-sustaining features combined with additional bonus of removing As from environment; (e) Potential of phytoremediation via phytoextraction, phytostabilisation, phytoaccumulation and phytovolatilisation.

1. Adaptive strategies for fighting As-stress

1. Strong antioxidative defense system

Arsenic triggers reactive oxygen species (ROS) generation in excessive level, which

results in oxidative damage of cell membranes, proteins, and nucleic acids, if not efficiently detoxified. There are several enzymatic (e.g. CAT, SOD, APX, GPX, GR) and non-enzymatic (e.g. ascorbate, glutathione, carotenoids, flavonoids, and phenolic compounds) role players of antioxidative defense in pteridophytes to overcome or mitigate oxidative stress.

2. Efficient transport and detoxification system

Pteridophytes utilize various transporter system for receiving As from environment based on concentration and available form [As(V), As (III) or organic form]. Accumulated As gets efficiently translocated from root to above-ground part and becomes compartmentalized in cell vacuole after detoxification.

3. Adaptation against herbivores

Pteridophytes become unappealing to grazers because they produce defensive secondary metabolites compounds like phenolics and alkaloids. They are used effectively to perform phytoremediation of toxic metal(loid)s.

4. Adaptation at molecular level

Well known As hyper-accumulator *Pteris vittata* possesses the ACR3 gene, responsible for production of a protein, responsible for transfer of arsenous acid into vacuoles. This gene is absent in angiosperms, helps the Pteridophytes safely store arsenic within its cells.

2. As accumulation by pteridophytes

Certain Pteridophytes have evolved to take, tolerate, compartmentalize and store As from environment into their root, rhizome, frond without suffering from its toxic effects. Continuous search for new As-hyperaccumulating pteridophyte species is going on. A comprehensive table (Table 1) illustrates an overview of As accumulating pteridophytes.

Table 1: List of As-accumulating pteridophytes used for environmental remediation

Plant species	As treatment	Duration	As in plant biomass (dry wt.)	References
<i>Pteris vittata</i>	As contaminated soil (400 mg kg ⁻¹)	6 weeks	6,805 mg kg ⁻¹ in frond	(Ma et al., 2001)
	As spiked soil (1500 mg kg ⁻¹)	6 weeks	22,630 mg kg ⁻¹ in frond	
<i>Pteris cretica parkerii</i>	As spiked soil (100 mg kg ⁻¹)	2 months	2493 mg kg ⁻¹ in frond	(Meharg, 2003)
<i>Equisetum hyemale</i>	As spiked soil (100 mg kg ⁻¹)	2 months	86.2 mg kg ⁻¹ in frond	
<i>Selaginella caulesens</i>	As spiked soil (100 mg kg ⁻¹)	2 months	26.3 mg kg ⁻¹ in frond	

Table Cont...

Plant species	As treatment	Duration	As in plant biomass (dry wt.)	References
<i>Adiantum capillus veneris</i>	As spiked soil (600 mg kg ⁻¹)	6 months	~2000 mg kg ⁻¹ in frond ~260 mg kg ⁻¹ in root	(Singh <i>et al.</i> , 2010)
<i>Nephrolepis exaltata</i>	As spiked soil (200 mg kg ⁻¹)	20 weeks	~4000 mg kg ⁻¹ in whole plant	(Slonecker <i>et al.</i> , 2009)
	As spiked hydroponic solution (10 µg g ⁻¹)	6 weeks	110 µg g ⁻¹ in frond	(Wongkongkatep <i>et al.</i> , 2003)
<i>Pityrogramma calomelanos</i>	As spiked hydroponic solution (10 µg g ⁻¹)	6 weeks	4616 µg g ⁻¹ in frond	

Chinese brake fern (*Pteris vittata* L.) the first known As hyper-accumulator, can accumulate 15,861 ppm of As in its above-ground parts, while growing in As-spiked soil (1,500 mg kg⁻¹) only after two weeks (Ma *et al.*, 2001). Eleven other species of Pteridaceae family have been

reported as As-hyperaccumulators (H.B. Wang *et al.*, 2007). Aquatic plants *Myriophyllum propinquum* and *Callitriche stagnalis* have been reported as As accumulator, with concentration exceeding 1g kg⁻¹ dry weight (Favas *et al.*, 2016).

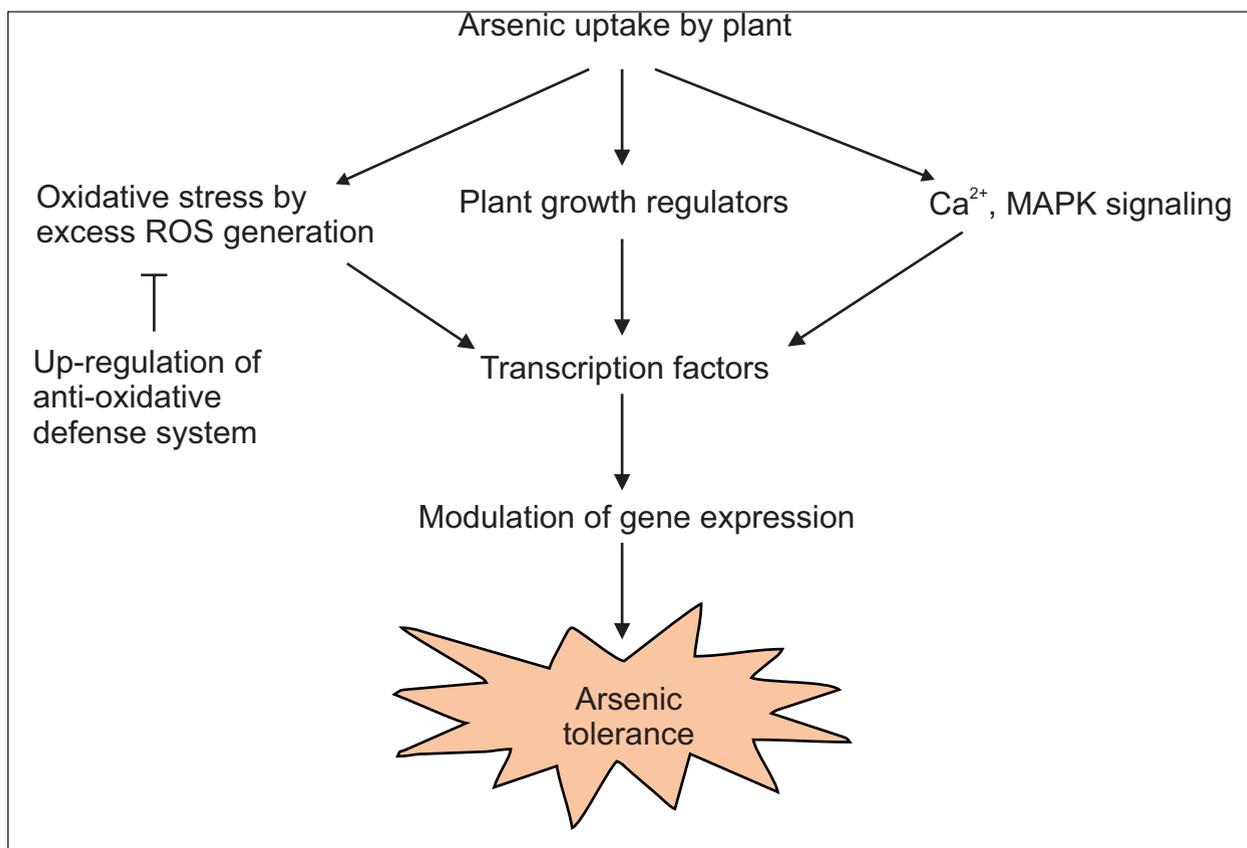


Figure 1: Transcription factors contribute significantly to As-stress tolerance by regulating the expression of stress-responsive genes

1. Arsenate uptake

Major fraction of As in soil remains in the form of As(V) under aerobic environments. Since As(V) (arsenate, AsO₄³⁻) is a structural and chemical analogue of inorganic phosphate (P_i; PO₄³⁻), it takes entry into root via Pi transporter through members of the Pht1 subfamily within the larger phosphate transporter (Pht) superfamily (Shi *et al.*, 2019).

Molecular studies have identified several Pht1 phosphate transporters involved in arsenate (AsV) uptake in *Pteris vittata*, particularly PvPht1;3, PvPht1;4, and PvPht1;6, which play key roles in facilitating the entry of arsenate into root cells (Y. Cao *et al.*, 2019). In hydroponic system with increased phosphate concentration As(V) uptake decreases in *P. vittata* while As(V) uptake increases under phosphorus-deficient

conditions (Fu *et al.*, 2017). But conflicting results came from some studies which showed that external amendment of phosphorus in soil increased As accumulation in *Pteris vittata*, primarily due to phosphorus-mediated release of As(V) in soil particles, thereby enhancing its bioavailability. Recent genomic studies revealed that PvPht1;3 functions as high affinity AsV transporter, facilitates As(V) absorption, translocation, accumulation in shoot when expressed heterologously in tobacco (Y. Cao *et al.*, 2019; Sun *et al.*, 2022). This is stronger As transport from soil to root mediated by PvPht1;4 (X. Li *et al.*, 2020). In anaerobic (mainly in flooded condition) As(III) in rhizospheric soil, is taken up by plants root through the aquaglyceroporin channels in the form of neutral As(OH)³ (Han *et al.*, 2017). A tonoplast intrinsic aquaporin protein PvTIP4; 1 in *P. vittata* root is upregulated in the presence of As(III) which in turn helps in As(III) accumulation (Z. He *et al.*, 2016). Two inositol transporter AtINT2 or AtINT4 are involved in As(III) loading in phloem and transport to seed of *Arabidopsis thaliana* (Duan *et al.*, 2015). As(III) uptake in higher plants is mediated by nodulin 26-like intrinsic proteins (NIPs) subfamily (e.g. PsPvH in *Pteris vittata* known as under aquaporins) (Fox *et al.*, 2017). As(III) is transported through OsLsi1 (also known as OsNIP;1) transporter in rice root along with soluble silicic acid (Bakhat *et al.*, 2017; Moulick *et al.*, 2023). Methylated As species in soil like monomethylarsonic acid [MMA(V)] and dimethylarsinic acid [DMA(V)] get entry into the *P. vittata* root through P_i transporter but less efficiently unlike As(V). While the trivalent form of aforesaid As species are more effectively enter cells via aquaglyceroporins.

2. Arsenate to arsenite reduction and efflux mechanism

Unlike hyperaccumulator fern *P. vittata*, other As non-hyperaccumulator pteridophytes rapidly convert As(V) to As(III). As(III) is chelated through cytosolic thiols specially by phytochelatins, *metallothionein* proteins or effluxed to the external environment. However, in As overloading condition roots of *P. vittata* exhibit significant As(III) efflux, which serves as an As detoxification strategy, otherwise As(III) would be readily loaded in xylem for upward translocation to shoot (Han *et al.*, 2016).

The key enzyme, ACR2 arsenate reductase regulates As(V) to As(III) conversion in plants. OsACR2, AtACR2, HlACR2, PvACR2

are the arsenic reductase enzyme found in rice, *Arabidopsis thaliana*, *Holcus lanatus*, and *Pteris vittata* respectively. However, recent research indicates that ACR2 is not solely responsible for As(V) to As(III) conversion in *Arabidopsis thaliana* while a new arsenate reductase enzyme named High Arsenic Content 1 (HAC1) or Arsenate Reductase QTL1 (ARQ1) has been reported as operating vital role in arsenate reduction in plants (F. Zhao *et al.*, 2023). PvHAC1 and PvHAC2 are two novel genes identified in *P. vittata* which predominantly expressed in rhizomes and frond respectively, but not in root. The distinct expression profiles of HACs in *P. vittata*, along with other evidences, indicate that arsenate reduction may primarily takes place in the rhizomes that facilitates rapid xylem loading of arsenite and subsequent translocation into fronds (X. Li *et al.*, 2020).

3. As translocation

Accumulated As is efficiently translocated to above ground parts of hyperaccumulating pteridophytes. Non-hyperaccumulators with translocation factor <1 are not be able to translocate As in above ground parts with maximum As concentration in root. The preferential translocation of arsenite from the roots to the fronds results in a markedly higher proportion of arsenite in the fronds than in the roots of *P. vittata* (F. Zhao *et al.*, 2023).

ACR3 (Arsenic Compounds Resistance 3) gene has been identified recently in yeast with no similarities with ACR2 gene (Y.S. Chen *et al.*, 2015). ACR3 is found in bacteria, moss, ferns, fungi but absent in angiosperms. Four ACR3 genes (PvACR3, PvACR3;1, PvACR3;2, and PvACR3;3) are identified in *P. vittata*. In hydroponic system treated with 5 µM As(III) or As(V). PvACR3 transgenic *Arabidopsis* sp. has exhibited 40 to 75-fold higher As translocation factors with 25 to 34 fold higher As concentration in shoot.

More recently, (H. Yan *et al.*, 2022) a new plasma membrane protein named PvAsE1, a member of the Solute Carrier Family 13 (SLC13)-like family has been identified. PvAsE1 functions as a plasma membrane As(III) efflux transporter that facilitates As(III) xylem loading from root parenchyma cells, assisting in its long-distance translocation.

4. As detoxification mechanisms

As non-hyperaccumulators with TF<1 protect their photosynthetic machinery by

preventing efficient translocation of As from root to frond (Y. Chen *et al.*, 2013). However, As hyperaccumulators like *P. vittata* employs a cascade of As detoxification mechanisms to cope up with excess As in its aerial parts. One of the key mechanisms is to sequester As(III) in cellular vacuoles of fronds that facilitates detoxification, storage, and hyperaccumulation of As. Significant As(III) sequestration occurs in *P. vittata* mediated by PvACR3;1 and PvACR3;3 located in the tonoplast (J. X. Chen *et al.*, 2021; C. Wang *et al.*, 2018).

Accumulated As in plants creates oxidative burst through the generation of reactive oxygen species (ROS). Excess ROS formation disrupts normal cellular and metabolic process ultimately cell death. Plants along

with pteridophytes have evolved several antioxidative defense system that helps in maintaining cellular optimal titer of ROS. Enzymatic [catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (GPX)] and non-enzymatic antioxidants [Ascorbate, Carotenoids, glutathione (GSH, GSSG), phytochelatins (PC)] play the key role in cellular antioxidative defense system. In As hyperaccumulators enzymatic and non-enzymatic antioxidants are playing more vigorously than that of non-hyperaccumulators (Praveen & Pandey, 2020). However, studies have shown that once the fronds reach their maximum As accumulation capacity, they undergo senescence and eventually abscise from the plant (Tu & Ma, 2002).

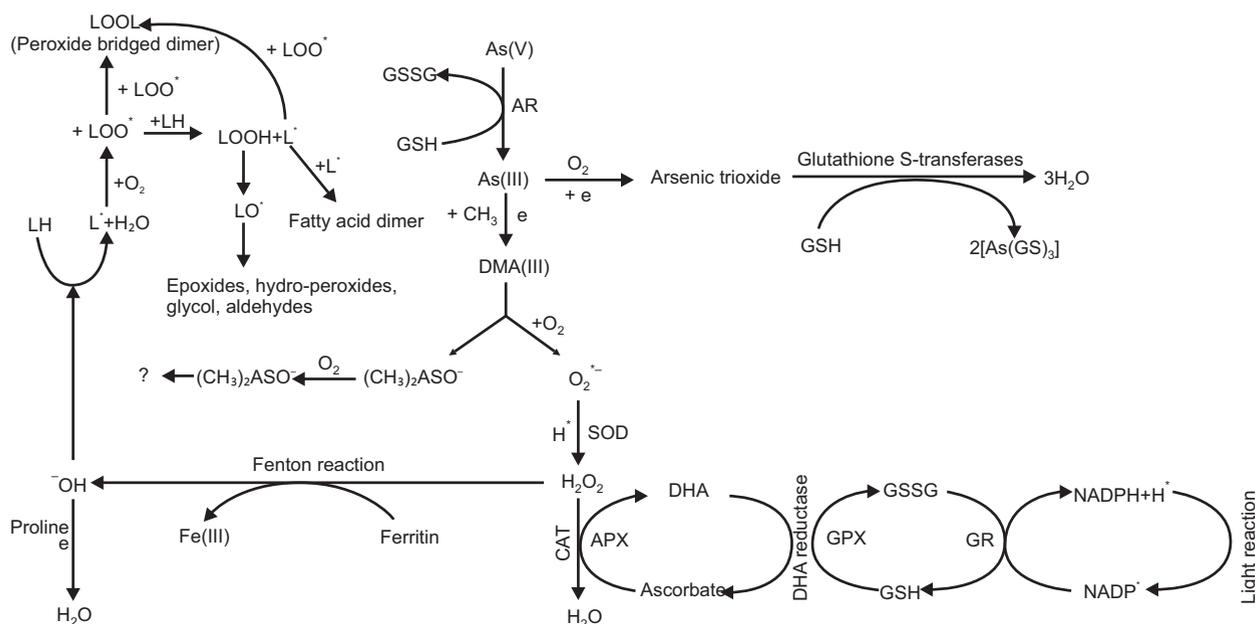


Figure 2: Arsenic detoxification pathway via cellular antioxidative defense system in pteridophytes, APX - ascorbate peroxidase, CAT - catalase, DHA - dehydroascorbate, DMA - dimethylarsenate, GPX - glutathione peroxidase, GR - glutathione reductase, GSH - reduced glutathione, GSSG - oxidized glutathione, LH - lipid, L* - lipid radical, LO* - lipid alkoxy radical, LOO* - lipid peroxy radical, LOOL - lipid peroxide, LOOH - lipid hydroperoxide, NADP - oxidized nicotinamide adenine dinucleotide phosphate, NADPH + H⁺ - reduced nicotinamide adenine dinucleotide phosphate, SOD - super oxide dismutase.

ENHANCEMENT OF PHYTOREMEDIATION OF AS

Enhancement of As phytoremediation helps to improve the efficiency of As removal from contaminated soils, which may be achieved by following methods.

1. Microbes-assisted phytoremediation

Rhizospheric microbial communities particularly bacteria and fungi enhance the potential of As sequestration and mitigation of As-induced oxidative stress in plants. Rhizospheric

microorganisms help to convert As into less toxic forms through redox reactions and immobilize it within their biomass which modify As bioavailability and enhance uptake by root (C. Yang *et al.*, 2022). The As transformation processes employed by rhizospheric microorganisms include As(V) reduction, As(III) oxidation, methylation of inorganic As into various n-methylated arsenic compounds. Plant growth-promoting rhizobacteria (PGPR) enhance plant growth and arsenic tolerance by producing siderophores that complexed

with As, phytohormones that promote plant growth, and enzymes that improve As-induced stress resistance. Recent studies have shown that inoculation of *Alcaligenes* sp. enhances As bioavailability and improves the overall remediation efficiency of the fern *P. vittata* by promoting As(V) reduction. Inoculating five potent As reducing bacterial strains, namely *Rhodococcus* sp. TS1, *Delftia* sp. TS41, *Delftia* sp. TS33, *Comamonas* sp. TS37, and *Streptomyces lividans* PSQ22 improved As accumulation in *P. vittata* by 53%, As uptake by 44%, and reduced As leaching in soil by 29 to 71% (Q. Yang et al., 2012).

2. Nano-phytoremediation

Nano-phytoremediation is a relatively new emerging branch of phytoremediation. In this technique engineered nanomaterials are used to improve As phytoremediation along with plant growth, stress tolerance, and other contaminants uptake from soil and water. The application of nanoparticles (NPs) such as TiO₂, ZnO, Au, and Si have been reported to alleviate metal toxicity significantly in *Oryza sativa* (Shukla et al., 2023; Wu et al., 2021), *Glycine max* (Y. Li et al., 2020), *Zea mays* (Lian et al., 2020). As remediation can be enhanced in *Isatis cappadocica* by application of salicylic acid-based NPs (Souri et al., 2017) whereas As stabilization is improved in *Helianthus annuus* by Zn nanoparticles (Vítková et al., 2018). Nano-TiO₂ application (4g L⁻¹) in *Vigna radiata* alleviates As toxicity (Katiyar et al., 2020) while at lower dose (1g L⁻¹) reduces As bioaccumulation in rice by 40 - 90% (Wu et al., 2021). Application of ZnO nanoparticles in rice helps to mitigate As-toxicity through up regulation of phytochelatin production and vacuolar sequestration of AsIII-PC complex (Shukla et al., 2023; S. Yan et al., 2021).

3. Biotechnological approach for enhancement of As phytoremediation

Transgenic As tolerant plants that can accumulate or detoxify As from environment have been developed by genetic engineering (Gunarathne et al., 2019; Lwanga et al., 2025). These genetically modified plants are designed for higher As uptake, efficient translocation from roots to fronds, transformation of As into less toxic forms, and improved arsenic tolerance (S. Thakur et al., 2019). In pteridophytes the uptake and transport of As(V) and As(III) are facilitated mainly by P_i transporters (PHTs)

and membrane intrinsic proteins (MIPs), respectively. Employing new transgenic approach where MIPs and PHTs genes are overexpressed could bring an enhanced As uptake and translocation, resulting in greater As accumulation in the engineered plants (Preetha et al., 2023).

A transgenic *Arabidopsis thaliana* has been developed by expressing PvACR3 gene which encodes an As(III) antiporter, under the control of the CaMV promoter, which results in 7.5 fold increase in As accumulation over the wild type. This transgenic *A. thaliana* has tolerance to 80 μm arsenite and 1200 μm arsenate which are lethal dose for wild type *A. thaliana* (Y. Chen et al., 2013).

Two transgenic rice varieties Nipponbare and Kasalath with gene OsPT8 mutation exhibit 33%-57% reduction in As(V) uptake than wild type (P. Wang et al., 2016). Mutations in the As transporters OsPht1:8 and Lsi 1/2 in rice has been found to significantly lower As uptake in the plants, consequently decreasing its accumulation in the rice grains (Y. Chen et al., 2017).

4. Amendments used to increase As phytoremediation efficiency

As phytoremediation efficiency of pteridophytes can be enhanced through application of phosphate, As-chelating agents, biochar etc. These amendments help to modify As bioavailability, uptake, transport, and also promote plant growth thereby increasing overall As remediation potential.

The application of plant growth regulators such as indole-3-acetic acid (IAA) has been found to enhance As uptake and growth in *Pteris cretica* var. *nervosa*, IAA treatment increased antioxidant enzyme activities, particularly superoxide dismutase (SOD) and peroxidase (POD), which suggest improved antioxidative activity (S. He et al., 2017).

The application of 20 mM calcium acetate improved drought tolerance and increased shoot As concentration by 165% and total As uptake by 55.1% in *P. vittata* (G. Wang et al., 2023). CaCO₃ treatment increased As accumulation in *Pteris vittata* fronds by 39% (up to 1460 mg kg⁻¹) compared to the CaCl₂ + As treatment, while reducing MDA by 16% (Y. F. Yang et al., 2025).

Application of 2.5 mmol kg⁻¹ oxalic acid (OA) significantly enhanced *P. vittata* growth and arsenic accumulation in multi-metal(loid) contaminated soil, increasing plant As uptake by 44.2% compared to the control after 20 days (Liang *et al.*, 2019).

In P limiting condition As accumulation is increased in pteridophytes whereas high P concentrations lower As absorption due to competitive inhibition at the root surface.

P. vittata grown in insoluble 2mM Ca-phytate or phosphate rock with 50 µM As in hydroponic system increased As accumulation (275–384% higher in fronds) whereas higher soluble P in medium reduce As accumulation capacity. An increase in phosphate concentration in the nutrient solution significantly reduced arsenic accumulation in both roots and shoots, indicating that phosphate competes with arsenate for uptake sites in *Pteris vittata* (Hu *et al.*, 2025).

Application of rice straw derived biochar (1.5% W/W) in As contaminated soil increased labile As in the soil, therefore accumulation reaching up to 350 mg kg⁻¹ in *P. vittata*, which is two times higher than control (Z. Y. Liu *et al.*, 2023).

Application of the chelating agent EDTA (ethylenediaminetetraacetic acid) resulted in a two-fold increase in shoot As accumulation in hydroponically grown *Pityrogramma calomelanos* after 6 months, exposure to 10 mg L⁻¹ As (disodium hydrogen arsenate). The shoot As concentration increased from 4,616 µg⁻¹ in the absence of EDTA to 7,290 µg⁻¹ following EDTA treatment (Wongkongkatap *et al.*, 2003).

CONCLUSIONS

It is well established that pteridophytes particularly *Pteris vittata* and related ferns play a crucial role as natural scavengers of soil As, which is attributed to their significant ability to uptake, translocate, and sequester As in above ground tissues. Physiological, molecular and environmental factors interplay and govern the efficiency of phytoremediation. In contrast to other plant groups pteridophytes are less economically important, herbaceous, fast-growing and generally non-edible, making them more suitable candidates for phytoremediation of As-contaminated soils. Their strong antioxidative defense helps reduce As-induced oxidative stress

by scavenging reactive oxygen species and maintaining cellular redox balance. However, further detailed investigations and pilot-scale studies are essential to optimize and validate the sustainable use of pteridophytes for large-scale arsenic bioremediation in affected environments. In the future, more research should focus on elucidating how *P. vittata* benefits from As to bridge the existing knowledge gap, while simultaneously exploring and identifying new As-hyperaccumulating pteridophyte species for improved phytoremediation potential with global acceptance.

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Standard journal article

1. Flink H, Tegelberg Å, Thörn M, Lagerlöf F. Effect of oral iron supplementation on unstimulated salivary flow rate: A randomized, double-blind, placebo-controlled trial. *J Oral Pathol Med* 2006; 35: 540-7.
2. Twetman S, Axelsson S, Dahlgren H, Holm AK, Källestål C, Lagerlöf F, *et al.* Caries-preventive effect of fluoride toothpaste: A systematic review. *Acta Odontol Scand* 2003; 61: 347-55.

Article in supplement or special issue

3. Fleischer W, Reimer K. Povidone-iodine antiseptics. *State of the art. Dermatology* 1997; 195 Suppl 2: 3-9.

Corporate (collective) author

4. American Academy of Periodontology. Sonic and ultrasonic scalers in periodontics. *J Periodontol* 2000; 71: 1792-801.

Unpublished article

5. Garoushi S, Lassila LV, Tezvergil A, Vallittu PK. Static and fatigue compression test for particulate filler composite resin with fiber-reinforced composite substructure. *Dent Mater* 2006.

Personal author(s)

6. Hosmer D, Lemeshow S. *Applied logistic regression*, 2nd edn. New York: Wiley-Interscience; 2000.

Chapter in book

- Nauntofte B, Tenovuo J, Lagerlöf F. Secretion and composition of saliva. In: Fejerskov O, Kidd EAM, editors. *Dental caries: The disease and its clinical management*. Oxford: Blackwell Munksgaard; 2003. pp 7-27.

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- World Health Organization. *Oral health surveys - basic methods*, 4th edn. Geneva: World Health Organization; 1997.

Reference from electronic media

- National Statistics Online – Trends in suicide by method in England and Wales, 1979–2001. www.statistics.gov.uk/downloads/theme_health/HSQ_20.pdf (accessed Jan 24, 2005): 7-18. Only verified references against the original documents should be cited. Authors are responsible for the accuracy and completeness of their references and for correct text citation. The number of reference should be kept limited to 20 in case of major communications and 10 for short communications.

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