PRODUCTION OF BIOCEMENT BY USING LIMESTONE AND AGRICULTURE WASTE AS SUBSTRATE FROM FUNGAL ISOLATES

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

Microbiology

By

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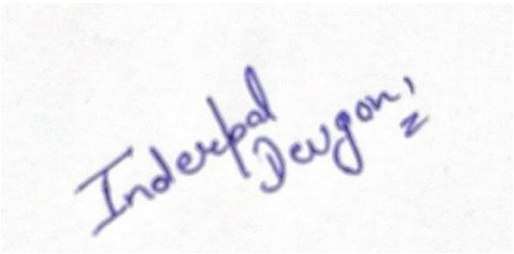
2025

DECLARATION

I, hereby declare that the presented work in the thesis entitled "Production of Bio-cement by using limestone and agriculture waste as the substrate from fungal isolates" in fulfilment of the degree of **Doctor of Philosophy (Ph. D.)** is the outcome of research work carried out by me under the supervision Prof. Arun Karnwal, working as Professor, in the School of Bioengineering and Biosciences of Lovely Professional University, Punjab, India. In keeping with the general practice of reporting scientific observations, due acknowledgments have been made whenever the work described here has been based on the findings of other investigators. This work has not been submitted in part or full to any other University or Institute for the award of any degree.



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CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled "Production of Bio-cement by using limestone and agriculture waste as the substrate from fungal isolates" submitted in fulfilment of the requirement for the reward of degree of Doctor of Philosophy (Ph.D.) in the School of Bioengineering and Biosciences, is a research work carried out by Inderpal Devgon, 12008960, is Bonafide record of his/her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

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ABSTRACT

With the increasing demand for sustainable construction materials, microbial-induced calcite precipitation (MICP) offers an eco-friendly approach for bio-cement production. However, most research has concentrated on bacterial MICP, leaving fungi applications underexplored. This thesis addresses this gap by investigating fungi MICP using urease-positive fungi isolated from alkaline soils in Punjab, specifically assessing their bio-cement production potential using limestone and agricultural wastes like rice straw as substrates.

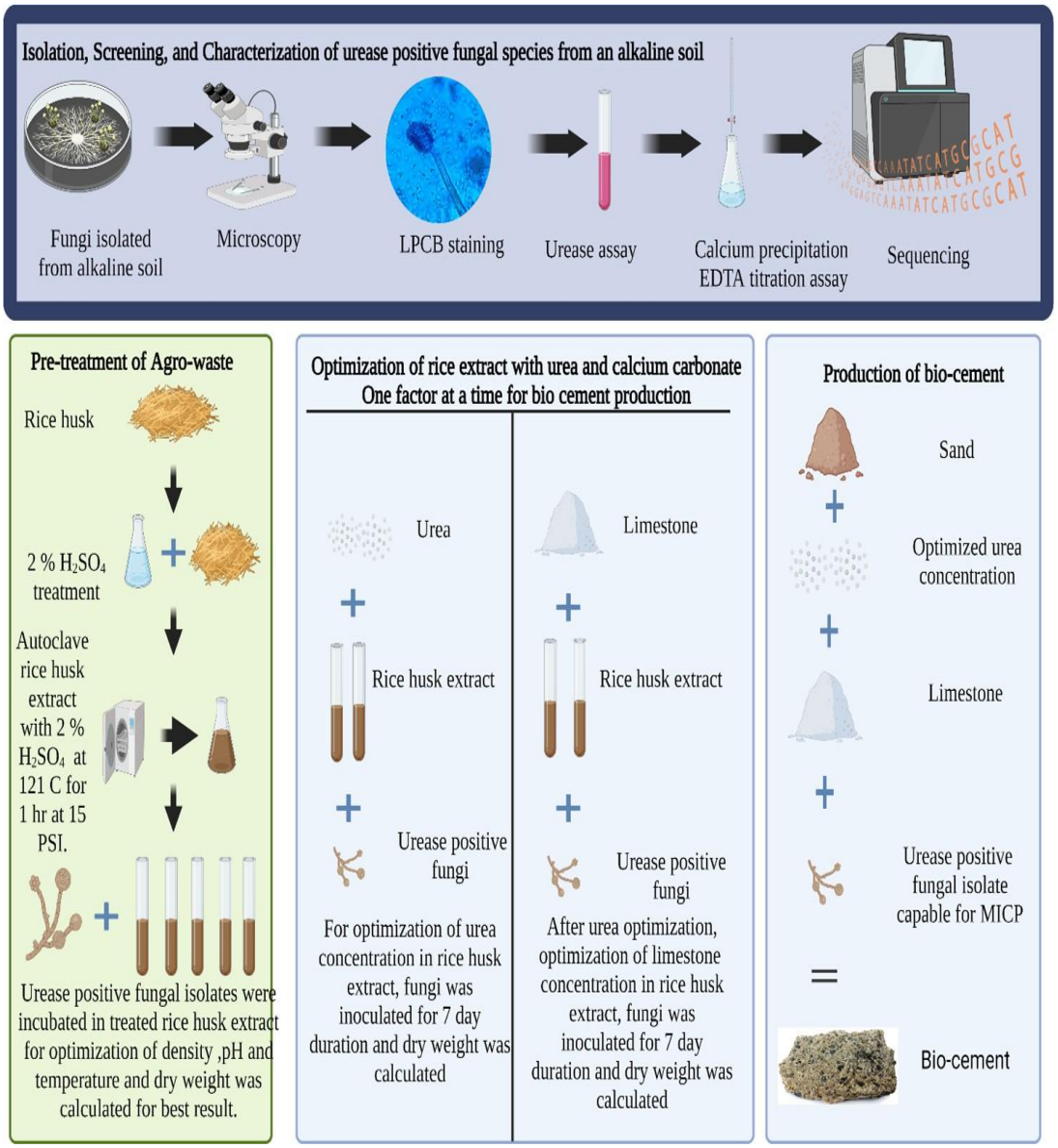
The study isolated 46 fungi strains from alkaline soils across regions in Punjab, including Phagwara, Moga, Muktsar, and Amritsar, which were screened for urease activity, an essential enzyme for MICP. Among these, three isolates, S1 (3), S4 (9), and S6 (9), demonstrated high urease activity. Quantitative assays revealed that S1 (3) had the highest urease production at 12 µg/ml, followed by S4 (9) with 10 µg/ml, and S6 (9) with 9 µg/ml, indicating strong calcite precipitation potential across the selected strains.

Cultivation of these isolates on acid-pre-treated rice straw substrates further optimized calcite formation. The 2% Sulphuric acid (H_2SO_4) treatment of rice straw enhanced nutrient availability, resulting in improved calcite precipitation rates. S1 (3) showed a 35% improvement in calcite deposition, while S4 (9) and S6 (9) showed enhancements of 30% and 28%, respectively. Fourier-transform infrared (FTIR) spectroscopy confirmed crucial functional groups for calcite formation, while X-ray diffraction (XRD) analysis indicated consistent calcite peaks at 2θ values, verifying successful biomineralization by all three fungi isolates. Thermal stability and compressive strength assessments further validated the structural properties of the produced bio-cement. Thermogravimetric analysis (TGA) showed minimal thermal degradation, with samples of S1 (3), S4 (9), and S6 (9) exhibiting weight loss of 10%, 11%, and 13%, respectively, up to 900°C, indicating high thermal stability across isolates. Compressive strength tests on bio-cement cubes revealed that cubes inoculated with S1 (3) achieved 12.6 MPa, S4 (9) reached 11.8 MPa, and S6 (9) achieved 10.9 MPa, all of which are comparable to the early-stage strengths of conventional cement, underscoring their potential in structural applications.

The durability and scalability of fungi bio-cement. Results demonstrated strong adhesion to crack surfaces. The compressive strength retention of up to 90% for S1 (3), 88% for S4 (9), and 85% for S6 (9) after 30 days in outdoor conditions noted, supporting their resilience and feasibility for real-world structural repair applications. Accelerated weathering tests confirmed further durability, with samples retaining over 85% of their initial strength after long-term environmental exposure.

Additionally, fungi bio-cement production demonstrated a significant reduction in CO_2 emissions, with emissions below 0.5 tons per ton of bio-cement, in contrast to the typical 0.9 tons in conventional Portland cement production. Life cycle analysis (LCA) suggested that fungi bio-cement could potentially lower carbon emissions by up to 40%, further contributing to its environmental viability and supporting a circular economy using rice straw waste as a substrate. The study provides evidence for the feasibility of fungi-induced MICP in bio-cement production, demonstrating that isolates S1 (3), S4 (9), and S6 (9) effectively enhance calcite precipitation on optimized substrates. The successful application of fungi bio-cement in pilot-scale wall crack repairs illustrates its potential for sustainable construction applications. Future research is suggested to increase biomineralization rates, explore long-term durability, and establish protocols for industrial-scale fungi bio-cement production as a viable building material.

GRAPHICAL ABSTRACT



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CHAPTER 1

INTRODUCTION

1.1 Overview

Cement, a crucial construction material known for its durability, strength, and resilience, is in high demand due to the growing population and subsequent construction needs. Cement manufacturing involves several sequential steps to produce the final product. Initially, raw materials such as limestone ($CaCO_3$), clay (SiO_2, Al_2O_3, Fe_2O_3), and other additives are extracted and crushed into fine particles (Lawrence et al.,1998). These materials are then proportionally mixed to form a raw meal, which is fed into a rotary kiln and heated to temperatures exceeding 1400°C. In the kiln, the raw materials undergo a series of chemical reactions: calcium carbonate decomposes into calcium oxide (quicklime) and carbon dioxide; silica, alumina, and iron oxide react to form various calcium silicates, aluminates, and ferrites. This process, known as clink erization, results in the formation of clinker nodules. The clinker is rapidly cooled and ground into a fine powder along with gypsum ($CaSO_4.2H_2O$) to control the setting time (Nuhu et al., 2020). The final product, Portland cement, comprises primarily of tricalcium silicate ($3CaO.SiO_2$), dicalcium silicate ($2CaO.SiO_2$), tricalcium aluminate ($3CaO.Al_2O_3$), and tetra calcium alumni ferrite ($4CaO.Al_2O_3.Fe_2O_3$), which contribute to its hydraulic properties and strength development upon hydration. However, the escalating global production of concrete and cement raises environmental concerns, as it leads to significant emissions of harmful gases from cement industries and other human activities involved in production. (Nuhu et al., 2020). Despite its advantages, cement also poses drawbacks such as early age cracking (Li et al., 2024), emission of toxic gases during production (Tarpani et al., 2024), high demand, and reliance on fossil fuels (Miller et al., 2024). Consequently, this manufacturing process contributes to environmental disturbances, highlighting the need for alternative materials or methods to address these issues. The growing global demand for sustainable construction materials has fuelled interest in innovative solutions that address environmental issues such as resource depletion and carbon emissions.

Bio-cement, produced through microbial-induced calcite precipitation (MICP), is a sustainable construction material that offers significant environmental benefits Unlike traditional cement, it requires fewer resources, and lower temperatures making it eco-friendly (Zhang et al., 2024). Bio-cement, primarily developed using bacteria, has self-

healing properties, allowing structures to repair microcracks over time, reducing maintenance costs, and extending durability (Onyelowe et al., 2024). Its innovative approach supports green construction practices, contributing to a lower carbon footprint and promoting sustainability in the building industry. Bio-cement is having more benefits as compared to conventional cement:

- Sustainability: Requires fewer natural resources and lower energy input compared to traditional cement, making it environmentally friendly (Tyagi et al., 2024).
- Eco-friendly Production: Utilizes microbial processes, reducing reliance on limestone and other natural materials (Nur et al., 2024)
- Lower Carbon Footprint: Produces less CO_2 emissions due to reduced energy requirements during production (Tyagi et al., 2024).
- Self-healing Properties: Can repair microcracks in structures over time, enhancing durability and reducing maintenance costs (Iqbal et al.,2021).
- Durability: Extends the lifespan of structures by maintaining integrity and reducing the need for frequent repairs (Nair et al., 2023).
- Versatility: Can be applied in various construction and infrastructure projects (Tyagi et al., 2024).
- Innovation in Construction: Promotes the use of microorganisms, fostering research and development in sustainable building materials (Armstrong et al., 2023).

1.2 Microbial Induced Calcite Precipitation (MICP)

Bio-cement production, which utilizes microorganisms like bacteria, algae, and fungi, presents a sustainable alternative to traditional cement. Unlike conventional cement production, which is energy-intensive and a major source of carbon dioxide emissions, bio-cement relies on biological processes to form calcium carbonate, a key component of the material (Gebru et al., 2021). Bio-cement can be produced using agricultural waste Implementing strategies such as the chemical treatment of rice straw to promote microbial growth and product formation could enhance its utilization. Bio-cement produced by bacteria represents a groundbreaking and sustainable approach to construction materials (Kumar et al., 2023). The key players in this innovative process

are bacteria, particularly strains like *Bacillus sp.* and *Sporosarcina pasteurii*, known for their unique ability to induce mineral precipitation. The production process typically involves introducing these bacteria into a mixture of calcium-rich materials such as limestone and a nutrient-rich medium. The bacteria, through metabolic activities, promote the conversion of calcium ions into calcium carbonate, leading to the formation of a cement-like substance (Achal et al., 2015). One remarkable feature of bacterial bio-cement production is its eco-friendly nature. Unlike traditional cement manufacturing, which involves high-energy processes and significant carbon emissions, bacterial bio-cement offers a more sustainable alternative. The microorganisms involved act as natural catalysts, reducing the need for high-temperature kilns and the release of greenhouse gases. This inherent environmental consciousness aligns with the global shift towards more sustainable and eco-friendly construction practices. Moreover, bacterial bio-cement holds promise for applications in soil stabilization, erosion control, and even self-healing concrete. Researchers are exploring the integration of bacteria into concrete mixtures, allowing for the repair of cracks and structural damage over time. This self-healing capability has the potential to extend the lifespan of structures and reduce maintenance costs. While bacterial bio-cement is still in the early stages of development, its potential impact on the construction industry is substantial. The ongoing research and experimentation in this field aim to optimize production processes, enhance material properties, and address scalability for broader adoption. As the demand for sustainable building materials grows, bacterial bio-cement emerges as a frontrunner in the quest for greener and more environmentally responsible construction practices.

Fungi, utilize agricultural waste as a rich source of nutrition, balancing carbon-to-nitrogen ratios, forming mycorrhizal associations to aid plant nutrient uptake, cultivating mushrooms on substrates like straw and sawdust, bio converting waste into biofuels and industrial products, and contributing to composting processes, sustainability while providing valuable resources for diverse applications (Cohen et al., 2001).

Microbial-induced calcite precipitation (MICP) can be facilitated by various microorganisms through mechanisms like denitrification (Pham et al., 2016),

dissimilatory sulphate reduction, ammonification, photosynthesis-induced alkalinity, iron reduction, methanogenesis, and urea hydrolysis (Castro-Alonso et al., 2019).

Denitrifying bacteria play a role in bio-cement production by utilizing nitrate (NO_3^-) or nitrite (NO_2^-) as alternative electron acceptors in anaerobic conditions. The reduction of nitrate or nitrite generates nitrogen gas (N_2) and results in an alkaline environment. The increased pH facilitates the precipitation of calcium carbonate through the reaction of carbonic acid with calcium ions (Pham et al., 2016).

Dissimilatory Sulphate Reduction Sulphate-reducing bacteria (SRB) contribute to bio-cement production through dissimilatory sulphate reduction. SRB utilizes sulphate (SO_4^{2-}) as a terminal electron acceptor in anaerobic conditions, producing hydrogen sulphide (H_2S). The generated H_2S reacts with calcium ions to form calcium sulphide (CaS), which then reacts with carbonate ions to produce calcium carbonate. (Castro-Alonso et al., 2019).

Ammonification involves the microbial conversion of organic nitrogen compounds into ammonia. Ammonia produced during this process can contribute to the alkalization of the environment, promoting the precipitation of calcium carbonate through reactions with calcium ions. (Castro-Alonso et al., 2019).

Photosynthesis-Induced Alkalinity Algae and cyanobacteria contribute to bio-cementation by inducing alkalinity through photosynthesis. During photosynthesis, these microorganisms consume carbon dioxide and release oxygen. The reduction in carbon dioxide concentration leads to an increase in pH, creating favorable conditions for calcium carbonate precipitation. (Castro-Alonso et al., 2019).

Some microbes can reduce iron (Fe^{3+}) under anaerobic conditions. This process produces ferrous ions (Fe^{2+}), leading to an increase in pH and creating conditions conducive to calcium carbonate precipitation. Organic Acid Production Certain microorganisms produce organic acids, such as citric acid and acetic acid, through metabolic processes. These acids contribute to the dissolution of minerals, releasing calcium ions and carbonate ions. The subsequent reaction of these ions leads to the formation of calcium carbonate. (Castro-Alonso et al., 2019).

Methanogenic archaea contribute to bio-cementation by producing methane gas in anaerobic conditions. The methane-producing process results in alkaline conditions, favouring the precipitation of calcium carbonate. Ammonium Oxidation Ammonium-oxidizing bacteria oxidize ammonia to nitrite or nitrate in a process known as nitrification. This oxidation reaction generates protons, leading to an increase in pH and creating conditions suitable for calcium carbonate precipitation. Sulfide Oxidation Some microorganisms are capable of oxidizing sulfide ions to produce sulfuric acid. This acidification process contributes to the dissolution of minerals and the release of calcium ions, facilitating the subsequent precipitation of calcium carbonate. (Castro-Alonso et al., 2019).

Urea hydrolysis is a key mechanism in bio-cement production, involving the enzymatic breakdown of urea by urease-producing microorganisms. Urease catalyses the conversion of urea into ammonia and carbon dioxide. The released ammonia reacts with calcium ions in the environment, leading to the precipitation of calcium carbonate. The most effective microbial pathway for MICP involves the hydrolysis of urea, catalysed by the enzyme urease (urea amidohydrolase, EC 3.5.1.5). During this process, urea is converted into ammonium and carbonate ions, leading to an increased saturation of calcium carbonate in the solution. This high saturation promotes the precipitation of calcium carbonate, typically as calcite. Microorganisms that can perform biomineralization are microbes involved in the nitrogen cycle. These bacteria produce urease enzymes that are responsible for the induction of calcium carbonate precipitation in the environment. The prime substrate for the precipitation is urea and later is calcium. With the help of urease, urea is broken down into ammonia and carbonic acid, and upon equilibrium with water forms a molecule of bicarbonate and two molecules of ammonia and hydroxide ions. This causes pH to increase in the bacteria and simultaneously causes bicarbonate equilibrium to shift towards the production of carbonate ions. Along with carbonate ions production, increased pH in the cell also pumps out the hydroxide ions which cause a further increase in the pH, hence, precipitating calcium as calcium carbonate. (Castro-Alonso et al., 2019).

$CO(NH_2)_2 + H_2O \rightarrow NH_2COOH + NH_3$

$NH_2COOH + H_2O \rightarrow NH_3 + H_2CO_3$

$H_2CO_3 \Leftrightarrow HCO_3^- + H^+$

$2NH_3 + 2H_2O \Leftrightarrow 2NH_4^+ + 2OH^-$

$HCO_3^- + H^+ + 2NH_4^+ + 2OH^- \Leftrightarrow CO_3^{2-} + 2NH_4^+ + 2H_2O$

$Ca^{2+} + Cell \rightarrow Cell\text{-}Ca^{2+}$

$Cell\text{-}Ca^{2+} + CO_3^{2-} \rightarrow Cell\text{-}CaCO_3^-$

1.3 Microbial-Induced calcite precipitation using Fungi

Fungal-mediated microbial-induced calcite precipitation (MICP) has emerged as a promising but relatively underexplored approach in biomineralization research. Filamentous fungi possess distinctive advantages for $CaCO_3$ precipitation, including extensive mycelial networks that act as nucleation sites and the ability to survive in harsh environments through spore formation (Ahmad et al., 2024; Van Wylick et al., 2024). Recent studies have demonstrated their potential in diverse applications, from enhancing the mechanical properties of sandy soils (Ahmad et al., 2024; Golovkina et al., 2024) to improving the durability of cementitious materials through self-healing mechanisms (Van Wylick et al., 2024; Tuyishime et al., 2025). Ureolytic fungi such as *Aspergillus niger*, *Trichoderma reesei*, and *Aspergillus iizukae* have been optimized to maximize $CaCO_3$ precipitation under controlled nutrient and calcium conditions, leading to significant improvements in compressive strength, crack sealing, and carbonation resistance (Ahmad et al., 2024; Alshaeer et al., 2024). Beyond civil engineering, fungal MICP has shown promise in environmental remediation, such as the immobilization of toxic heavy metals like Zn^{2+} and Cr^{6+} using marine *Rhodotorula* species (Awadeen et al., 2024). Research has also explored innovative applications, including mineralized mycelium bio-composites with enhanced mechanical performance (Ningsih et al., 2025). While these developments highlight the versatility and potential of fungal MICP, it is important to note that bacterial MICP has been studied for several decades and is therefore represented more extensively in the current literature, a trend reflected in the emphasis on bacterial systems within this thesis.

Fungi are well known for the production of urease enzyme (Adams et al., 1978), urease enzyme catalyzes the hydrolysis of urea, producing carbonate ions that combine with calcium to form calcium carbonate, replicating the cementation process (Goswami et al., 2019). This bio-mediated mineralization reduces the environmental impact of cement production by eliminating the need for energy-intensive kilns and incorporating eco-friendly substrates like agricultural waste to promote sustainability. Bio-cement leverages the natural ability of microorganisms to induce the precipitation of minerals. Researchers have less explored the MICP using fungi such as *Aspergillus sp.* (Ahmad et al., 2024), *Penicillium sp., Fusarium sp., Trichoderma sp.* (Khan et al., 2023) which play a significant role in bio-cementation by generating organic acids that dissolve minerals, releasing essential ions for cement formation. These fungi also contribute to the material's structural strength by acting as natural binders. The adaptability of microorganisms in bio-cement production offers numerous applications, from soil stabilization to sustainable construction, underscoring the potential and versatility of this emerging technology (Asghnarpour et al., 2024).

Benefits of Fungi Microbial-Induced Calcite Precipitation (MICP) (Zhang et al., 2024):

- Diverse Metabolic Pathways: Fungi offer diverse metabolic capabilities, potentially leading to more efficient calcite precipitation compared to bacteria.
- Improved Stability: Fungi can form extensive hyphal networks that enhance the structural stability of bio-cement, making it more durable.
- Tolerant to Harsh Conditions: Fungi are often more resilient in extreme environmental conditions, such as acidic or nutrient-poor environments, expanding the potential applications of bio-cement.
- Sustainable Resource: Fungi MICP still uses fewer natural resources and lower temperatures, contributing to the eco-friendliness of bio-cement.
- Potential for Novel Applications: Since fungi remain underexplored in MICP, they offer opportunities for developing new, innovative bio-cement technologies.
- Mycelium helps in binding soil particles which enhances the strength of bio-cement bricks,
- Fungus has more nucleation cites as compared to bacteria which will result in more urease enzyme production which is required in MICP.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Conventional cement

Cement production is a significant contributor to global carbon dioxide (CO_2) emissions, with substantial implications for climate change and environmental sustainability. The primary process involved in cement production is the calcination of limestone (calcium carbonate), which releases large quantities of CO_2 and a high requirement for energy (Figures 2.1 and 2.2) (Habert et al., 2011; Scrivener et al., 2016). This process occurs at high temperatures, requiring significant energy input, often derived from fossil fuels, further exacerbating CO_2 emissions. The cement industry is responsible for approximately 8% of global CO_2 emissions, highlighting its impact on the environment (Andrew, 2018). In 2018, global cement production emitted about 2.8 billion metric tons of CO_2, underscoring the scale of its environmental footprint. The production of one ton of cement typically generates about one ton of CO_2, making it one of the most carbon-intensive industrial activities (Barcelo et al., 2014).

The cement industry's contribution to climate change is significant due to the scale of its operations. Cement is a fundamental component of concrete, the most widely used construction material globally. This widespread use means that the environmental impact of cement production is far-reaching. In addition to CO_2 emissions, cement production also results in the release of other greenhouse gases (GHGs) and pollutants that can harm air quality and contribute to global warming (Mahasenan et al., 2003). For instance, nitrogen oxides (NO) and sulfur oxides (SO) are also emitted during cement production, further contributing to environmental degradation (Mishra et al., 2022). Recent studies have highlighted the urgent need for the cement industry to adopt more sustainable practices to mitigate its environmental impact. Innovations such as the use of alternative materials, carbon capture and storage (CCS) technologies, and the development of low-carbon cement are being explored as potential solutions (Mohamad et al., 2022). These approaches aim to reduce the carbon footprint of cement production while maintaining the material's essential properties and performance. For example, replacing traditional clinker with supplementary cementitious materials (SCMs) like fly ash, slag, and natural pozzolans can reduce CO_2 emissions by up to 40% (Nie et al., 2022).

The transformation of the concrete and cement industry is critical in addressing climate change challenges. Efforts to improve energy efficiency, utilize alternative fuels, and implement new technologies are crucial steps toward reducing emissions (Belaid et al., 2022). Advanced manufacturing techniques and the integration of renewable energy sources into cement production processes can also play a significant role in decreasing the industry's carbon footprint. Moreover, policies and regulations that promote sustainable practices and carbon reduction targets are essential for driving industry-wide changes (Miller & Moore, 2020). This is where bio-cement comes into play as an alternative. Bio-cement, produced through microbially induced calcite precipitation (MICP), offers a sustainable solution to traditional cement. By leveraging biological processes to induce the precipitation of calcium carbonate, bio-cement production can occur at ambient temperatures and pressures, significantly reducing CO_2 emissions and energy consumption. This innovative approach not only addresses the environmental challenges associated with conventional cement production but also utilizes waste materials as substrates, further promoting sustainability and resource efficiency.

Bio-cement, also known as bio-cement or biogenic cement, is a sustainable construction material produced through a process called microbially induced calcite precipitation (MICP). MICP involves the use of specific microorganisms, such as bacteria and fungi, to induce the precipitation of calcium carbonate, resulting in the formation of a cement-like material. The primary mechanism of MICP is the hydrolysis of urea by urease-producing microorganisms, which generates carbonate ions. These ions react with calcium ions present in the environment to form calcite, a stable form of calcium carbonate. This biogenic process mimics natural sedimentation and mineralization processes, making bio-cement an environmentally friendly alternative to conventional cement (Gebru et al., 2021).

The significance of bio-cement in sustainable construction is profound due to its potential to significantly reduce the environmental footprint of the construction industry. Traditional Portland cement production is a major contributor to global CO_2 emissions, accounting for approximately 8% of the total emissions. This is largely due to the high-temperature calcination process required to produce clinker, the key

ingredient in Portland cement. In contrast, bio-cement production can occur at ambient temperatures and pressures, leading to a substantial reduction in energy consumption and greenhouse gas emissions. By utilizing naturally occurring microorganisms, bio-cement offers a more sustainable method of cement production that aligns with global efforts to combat climate change (Yu et al., 2022). Moreover, bio-cement production can effectively utilize waste materials, such as agricultural residues and industrial by-products, as substrates for microbial growth. This not only addresses waste disposal issues but also promotes the recycling of organic waste, contributing to a circular economy. For instance, agricultural wastes like rice husks, straw, and corn cobs, which are rich in organic matter, can serve as nutrient sources for the microorganisms involved in MICP. This dual benefit of waste utilization and bio-cement production exemplifies an innovative approach to resource management and sustainability in construction (Qian et al., 2018).

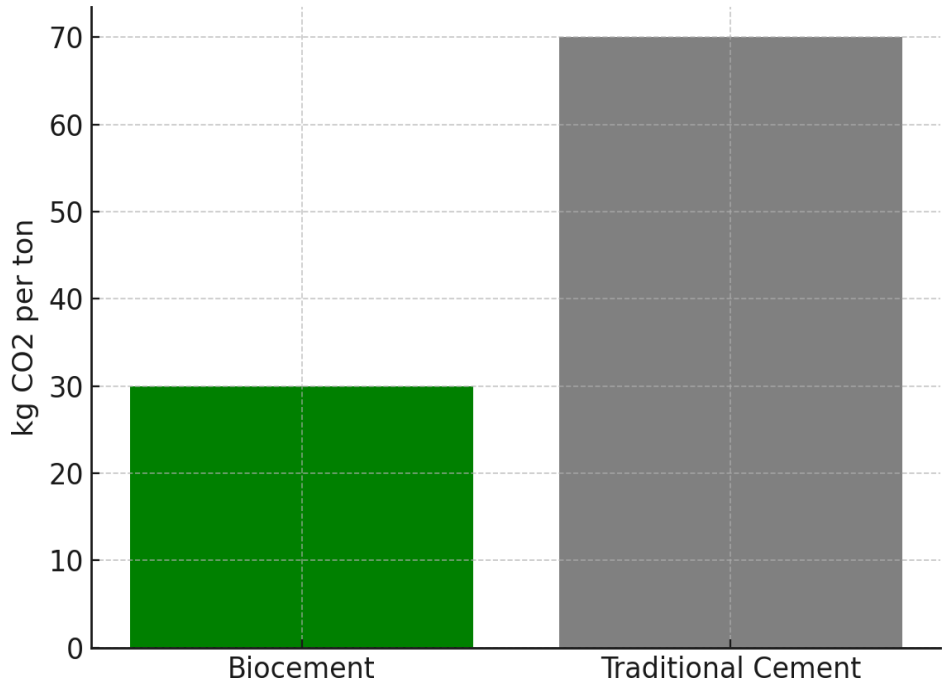


Figure 2.1: The CO_2 emissions associated with bio-cement and traditional cement production.

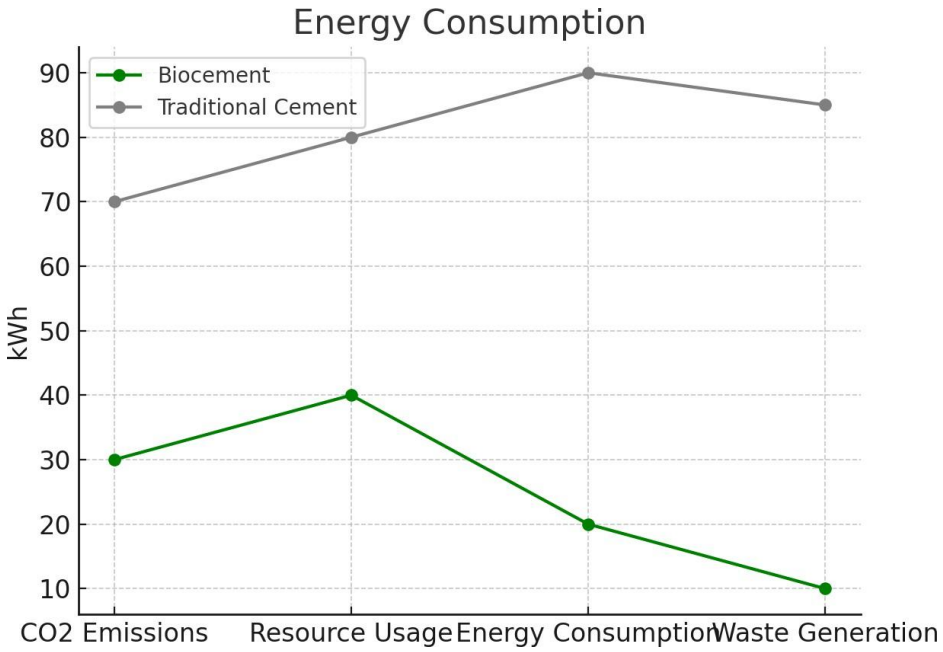


Figure 2.2: Energy use in the production of bio-cement versus traditional cement

In addition to environmental benefits, bio-cement also enhances the durability and longevity of construction materials. The microbially induced calcite precipitation process fills pores and cracks within the material, improving its structural integrity and resistance to weathering. This not only extends the lifespan of construction materials but also reduces the need for frequent repairs and maintenance, further decreasing the overall environmental impact. The enhanced properties of bio-cement-modified construction materials have been demonstrated in various studies, showing improved compressive strength and reduced permeability, which are critical factors in the performance of construction materials (Yu at el., 2022).

The concept of bio-cement, or the use of biological processes to create cementitious materials, has deep historical roots and has evolved significantly over time. The idea of using naturally occurring materials and biological processes for construction can be traced back to ancient civilizations. For instance, historical records indicate that the use of natural cements, which were derived from readily available organic and inorganic materials, was common in ancient China, India, and Rome. These early civilizations leveraged the natural bonding properties of materials such as lime and pozzolanic ash

to create durable structures, some of which have withstood the test of time (Su et al., 2023). The modern development of bio-cement, however, began more recently, with significant advancements occurring over the past few decades. In the mid-20th century, scientists began to explore the potential of using microorganisms to induce the formation of calcium carbonate, inspired by natural processes observed in marine environments and caves. This interest was driven by the need for more sustainable construction materials that could reduce the environmental impact of traditional cement production. Early research focused on understanding the biological mechanisms behind microbial-induced calcite precipitation (MICP) and identifying suitable microbial species for this process (Achal et al., 2014).

The 1990s and early 2000s saw a surge in research activity aimed at harnessing MICP for practical applications in construction. During this period, significant progress was made in isolating and cultivating urease-producing bacteria, such as *Sporosarcina pasteurii*, which are capable of precipitating calcium carbonate. Researchers developed laboratory-scale methods to test the effectiveness of these bacteria in cementing loose sand and soil particles, laying the groundwork for potential real-world applications (Achal et al., 2016). By the early 2010s, the field of bio-cement had expanded to include various types of microorganisms, including bacteria and fungi, capable of inducing calcite precipitation. Advances in genetic engineering and microbiology enabled scientists to enhance the calcite-producing capabilities of these microorganisms, further improving the efficiency and effectiveness of the bio-cementation process. During this period, China emerged as a leading hub for bio-cement research, driven by the country's rapid urbanization and the associated environmental challenges. Chinese researchers made significant contributions to the development of bio-cement technologies, focusing on both fundamental research and practical applications (Achal at el., 2014).

In recent years, bio-cement has moved from the laboratory to the field, with several successful full-scale applications demonstrating its potential. For example, case histories of microbial bio-cement applications for surface erosion control have shown promising results in stabilizing soil and reducing erosion in various environmental settings. These projects have provided valuable insights into the practical challenges

and opportunities associated with large-scale bio-cement implementation (Hodges et al., 2020). The evolution of bio-cement is marked by continuous innovation and interdisciplinary collaboration. Researchers from fields such as microbiology, materials science, civil engineering, and environmental science have worked together to refine bio-cement technologies and explore new applications. Current research focuses on optimizing the production process, scaling up bio-cement applications, and developing bio-cement formulations that can be tailored to specific construction needs. Additionally, there is growing interest in integrating bio-cement into the broader context of sustainable urban planning and eco-friendly building practices (Achal et al., 2016).

2.2 Microbial Induced Calcite Precipitation

Microbially Induced Calcite Precipitation (MICP) is a biotechnological process that utilizes the metabolic activities of certain microorganisms to induce the precipitation of calcium carbonate in various environments, offering a sustainable solution for soil stabilization, construction material reinforcement, and environmental remediation (Chuo et al., 2020). This innovative approach has garnered significant attention in recent years due to its potential to mitigate environmental degradation caused by traditional cement production and its associated CO_2 emissions (Fouladi et al., 2023).

The MICP process involves a series of biological and chemical reactions mediated by specific bacteria, primarily ureolytic microorganisms such as *Sporosarcina pasteurii*. These bacteria possess the enzyme urease, which catalyzes the hydrolysis of urea into ammonia and carbon dioxide. This initial step, known as urea hydrolysis, is fundamental to the MICP process as it provides the necessary precursors for calcium carbonate precipitation (Bhutang et al., 2020). The reaction can be represented as follows:

$$CO(NH_2)_2 + H_2O \rightarrow CO_2 + 2NH_3$$

The ammonia produced from urea hydrolysis subsequently undergoes hydrolysis to generate ammonium ions (NH_4^+) and hydroxide ions (OH^-). This process increases pH,

creating a favorable environment for carbonate ion formation (Chuo et al., 2020). The chemical reactions involved in ammonia hydrolysis are as follows:

$NH_3 + H_2O \rightarrow NH_4^+ + OH$

Once the pH of the surrounding environment rises, carbonate ions (CO_3^{2-}) are formed through the reaction between hydroxide ions and atmospheric carbon dioxide. These carbonate ions then react with calcium ions (Ca^{2+}) present in the environment to precipitate calcium carbonate ($CaCO_3$), the main constituent of bio-cement (Gebru et al., 2021). The precipitation reaction can be represented as:

$Ca^{2+} + CO_3^{2-} \rightarrow CaCO_3$

The precipitated calcium carbonate forms bonds between soil particles or within cracks in construction materials, resulting in bio-cementation and strengthening of the substrate (e Portugal et al., 2020). This process enhances the mechanical properties of the material, such as compressive strength and stiffness, while also reducing permeability and improving durability (Erdmann et al., 2023).

The MICP process can be implemented in various applications, including soil stabilization, construction material reinforcement, and environmental remediation. In soil stabilization, MICP can be used to improve the engineering properties of soil, such as shear strength and bearing capacity, by injecting bacterial solutions into the ground and allowing the bacteria to precipitate calcium carbonate, effectively cementing the soil particles together (Pacheco et al., 2022). This technique has been successfully applied in geotechnical engineering projects to prevent soil erosion, mitigate slope instability, and enhance the stability of embankments and foundations (Ali et al., 2023).

In construction materials, MICP can be employed to enhance the performance of concrete, mortar, and other cementitious materials by incorporating ureolytic bacteria and calcium sources into the mix design. The bacteria then induce calcium carbonate precipitation within the material matrix, resulting in improved mechanical properties and durability (Zhang et al., 2024). Additionally, MICP can be used for crack repair and self-healing of concrete structures, where bacteria are introduced into existing

cracks, and calcium carbonate is precipitated to seal the cracks and prevent further propagation (Fouladi et al., 2023).

MICP technology also holds promise for environmental remediation applications, such as the immobilization of heavy metals and the remediation of contaminated sites. By precipitating calcium carbonate in the presence of heavy metal ions, MICP can effectively encapsulate and immobilize contaminants, reducing their mobility and bioavailability in the environment (Bhutange et al., 2020). This approach has been explored for the remediation of contaminated groundwater, soil, and sediments, offering a cost-effective and environmentally friendly solution for addressing pollution issues (Chuo et al., 2020)

Fungi isolates play a significant role in bio-cement production, offering unique mechanisms and advantages that distinguish them from bacterial processes. Fungi, with their extensive hyphal networks and robust enzymatic capabilities, can induce calcium carbonate precipitation ($CaCO_3$) through several pathways, contributing to soil stabilization and material reinforcement.

One of the primary mechanisms by which fungi contribute to bio-cement production is through the secretion of organic acids. Fungi such as *Aspergillus niger* and *Penicillium chrysogenum* produce acids like citric acid and oxalic acid as metabolic byproducts. These acids react with available calcium ions in the environment to form compounds such as calcium citrate or calcium oxalate. Over time, these compounds can undergo further chemical reactions, transforming into calcium carbonate. The resulting calcium carbonate crystals enhance the mechanical properties of the soil or material they bind, contributing to increased stability and strength.

Additionally, fungi can precipitate calcium carbonate through the biomineralization process. Certain fungi species, like *Mucor* and *Trichoderma*, can influence the local pH and create microenvironments conducive to $CaCO_3$ precipitation. The fungi hyphae serve as nucleation sites, where calcium carbonate crystals form and grow. This mycelial network not only aids in the nucleation process but also distributes the precipitated $CaCO_3$ more uniformly throughout the material.

Research has shown that fungi induced bio-cement can improve the compressive strength and durability of treated soils. For example, studies have demonstrated that fungi treatments can increase soil compressive strength by up to 1.2 MPa, making it a viable alternative or complement to bacterial MICP. Furthermore, fungi isolates can operate under a wider range of environmental conditions compared to bacteria, including lower pH levels and different temperature ranges, providing greater flexibility in bio-cement applications.

2.3 Substrates for Bio-cement Production

2.3.1 Limestone as a Substrate

Limestone, a sedimentary rock rich in calcium carbonate ($CaCO_3$), remains one of the most widely investigated substrates for bio-cement production due to its global abundance, high $CaCO_3$ content, and accessibility (Zheng et al., 2015; Ghosh et al., 2018). While its composition typically includes 40–50% CaO alongside MgO, SiO_2, Al_2O_3, and Fe_2O_3, its bio-cementation performance is not uniform and is influenced by particle size, pretreatment, and application method. Direct use of aggregates provides a readily available calcium source and surfaces for microbial attachment, but studies suggest that increased surface area through powdering or pretreatments can significantly enhance reactivity (Zheng et al., 2015). Mechanical grinding consistently shows performance gains by increasing contact between limestone and microorganisms, yet the degree of benefit is size-dependent. Zheng et al., (2015) demonstrated a 30% higher precipitation rate for <100 µm particles, while Ghosh et al, (2018) linked 75 µm powders to a 25% rise in soil compressive strength compared to coarser material. These findings collectively indicate that finer particles improve bio-cement efficiency, though energy costs and equipment wear demand cost–benefit optimisation before large-scale adoption.Chemical treatments such as HCl and citric acid etching increase porosity and surface roughness, thereby boosting nucleation sites for $CaCO_3$ precipitation (Li et al., 2017; Wang et al., 2019). However, the literature highlights the need to balance enhancement with risks of over-dissolution and environmental impact from acid residues. Citric acid may present a greener alternative, but direct comparative studies under identical conditions are limited, leaving an evidence gap regarding optimal chemical agents for field use. Thermal treatments alter limestone microstructure and

phase composition to improve reactivity, with (Zhao et al., 2018) and (Sun et al., 2020) both finding that heating to around 600 °C optimized both surface area and precipitation rates. Still, high energy requirements and carbon emissions may offset these benefits, and life-cycle assessments are rarely reported in the reviewed studies. Application-focused research further reinforces limestone's versatility. (DeJong et al., 2006) and (Qabany et al., 2012) demonstrated major strength gains (150–200%) in soils, while (Achal et al., 2011) and (Ariyanti et al., 2012) showed improved mortar performance and reduced carbon footprint in tropical construction contexts. (Choi et al. 2016) extended its application to sustainable crack repair, validating limestone aggregate's compatibility with MICP for infrastructure maintenance. However, despite these successes, few studies directly compare limestone with alternative substrates under standardised protocols, limiting definitive conclusions about its relative efficiency in diverse environmental settings.

2.3.2 Agricultural Waste as a Substrate

Types of Agricultural Waste

Agricultural waste has emerged as a valuable resource for sustainable construction materials, including bio-cement production. Bio-cement, a biologically produced binder, is gaining attention due to its potential to reduce the carbon footprint of traditional cement production. Utilizing agricultural waste not only addresses waste management challenges but also provides an eco-friendly alternative to conventional cement. This essay discusses the various types of agricultural waste that can be used in bio-cement production, focusing on rice husks, straw, corn cobs, and other significant residues. However, only 40% of the natural resources are utilized for bio-cement production (Figure 2.3) (Gartner and Sui, 2018; Shi et al., 2006).

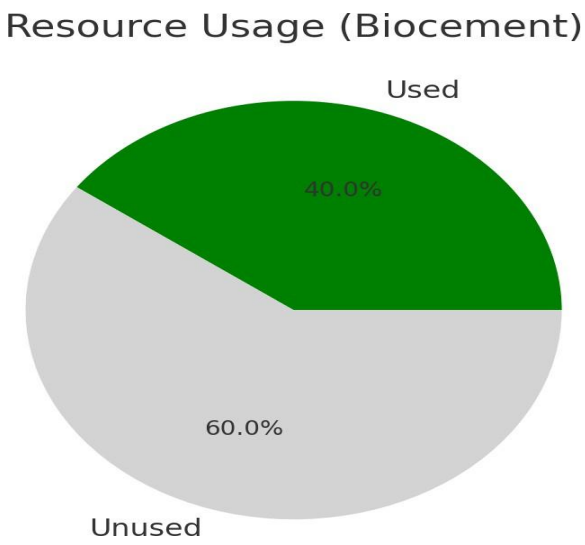


Figure 2.3: Pie chart representing the percentage of natural resources consumed during the production of bio-cement.

2.3.2.1 Rice Husks

Rice husks are the protective outer layers of rice grains, constituting about 20% of the weight of the harvested rice. They are one of the most abundant agricultural wastes globally, especially in Asia, where rice production is prevalent. Rice husks are rich in silica, which makes them particularly suitable for bio-cement production. When burned, rice husks produce rice husk ash (RHA), which contains up to 90% amorphous silica. This high silica content can be utilized as a supplementary cementitious material (SCM) in concrete, improving its strength and durability. Additionally, RHA enhances the pozzolanic reaction in cement, contributing to the overall performance of bio- cement. Studies have shown that concrete incorporating RHA exhibits reduced porosity and increased resistance to chemical attacks, making it a viable material for sustainable construction (Mehta, 2019).

2.3.2.2 Straw

Straw is another abundant agricultural residue, mainly composed of the stalks left after the harvesting of cereal grains such as wheat, barley, and rice. Straw has been traditionally used in construction, particularly in the production of straw-bale houses.

In bio-cement production, straw can be used as a fiber additive to improve the mechanical properties of the cement. The fibrous nature of straw provides reinforcement, enhancing the tensile strength and cracking resistance of bio-cement. Moreover, straw can be pyrolyzed to produce biochar, a carbon-rich material that can be incorporated into bio-cement as a filler or SCM. Biochar has the potential to reduce the density of the cement, making it lighter and more insulating, which is advantageous for energy-efficient buildings. The use of straw in bio-cement not only contributes to waste reduction but also promotes the sequestration of carbon, making the construction process more sustainable (Glaser et al., 2020).

2.3.2.3 Other Agricultural wates

Several lignocellulosic residues have shown potential for sustainable bio-cement production. Corn cobs, rich in cellulose, hemicellulose, and lignin, can be processed into biochar to enhance compressive strength, durability, and thermal insulation in cement, supporting circular economy principles (Kumar et al., 2018). Sugarcane bagasse, a high-cellulose byproduct of the sugar industry, improves flexural strength and toughness when incorporated as fibers, while its ash, rich in silica, serves as a supplementary cementitious material (SCM) that reduces CO_2 emissions and production costs (Cordeiro et al., 2012). Palm oil fuel ash (POFA), containing silica, alumina, and calcium oxide, enhances pozzolanic reactions, strength, and durability, while reducing hydration heat in large structures (Awal & Hussin, 2011). Coconut husks and shells yield biochar that improves compressive strength, durability, and insulation, with fibers providing tensile reinforcement (Gunasekaran et al., 2013). Peanut shells, with high porosity biochar, enhance mechanical and insulation properties, promoting waste valorization (Ibrahim et al., 2020). Banana leaves and stems, processed into fibers or biochar, improve tensile, flexural, and insulation properties, offering an eco-friendly solution for sustainable construction (Sathiparan et al., 2019).

2.3.3 Rice husk as a favored substrate

Rice straw is one of the most abundant agricultural residues in rice-growing regions, constituting roughly 40–50% of the rice harvest by mass, and is widely available at low cost (Srivastava et al., 2023). In many areas, it is underutilized or disposed of by open

burning, which contributes significantly to air pollution (Srivastava et al., 2023). Its high organic content—primarily cellulose and hemicellulose—makes rice straw an excellent carbon source to support microbial growth and metabolism (Cai et al., 2023; Srivastava et al., 2023). Utilizing this lignocellulosic waste as a substrate in microbial-induced calcite precipitation (MICP) therefore provides both a nutrient-rich medium for calcite-producing microbes and a sustainable use for an otherwise polluting agro-waste.

Economically, rice straw's abundance and waste status translate to very low material cost, enhancing the feasibility of biocement applications (Cai et al., 2023). Recent studies have confirmed its suitability in both bacterial and fungal MICP processes. For example, incorporating rice straw into ***Sporosarcina pasteurii***-based treatments not only improved $CaCO_3$ precipitation but also enhanced soil quality during bioremediation (Cai et al., 2023).

In summary, the use of rice straw as a biocement substrate is justified by its ubiquity, high cellulose-driven nutritive value for calcifying microbes, minimal cost, and potential for sustainable and economically viable biocement production (Cai et al., 2023; Srivastava et al., 2023).

Rice husk ash (RHA) has garnered considerable attention in the field of sustainable construction, particularly in bio-cement production. The scientific community has explored its potential to replace or supplement conventional cement due to its high silica content, which significantly influences the mechanical and durability properties of concrete. (Datta et al., 2024)

2.3.3.1 Silica Content and Pozzolanic Activity

Rice husk, when burned under controlled conditions, produces rice husk ash (RHA) with a silica content ranging from 85% to 90% in an amorphous form. This high silica content is crucial for the pozzolanic reaction, where silica reacts with calcium hydroxide in the presence of water to form an additional calcium silicate hydrate (C-S-H), the primary binding phase in concrete. Studies by (Mehta, 2019) have shown that the pozzolanic activity index of RHA can reach up to 100% at 28 days, compared to 75% for other conventional pozzolanic materials like fly ash. This high activity makes RHA a highly effective supplementary cementitious material (SCM).

2.3.3.2 Mechanical Properties Enhancement

The inclusion of RHA in cement has been shown to enhance various mechanical properties of concrete. According to research by Hwang and Chandra, 2018, the compressive strength of concrete mixtures containing 10% to 20% RHA as a replacement for ordinary Portland cement (OPC) increased by 10% to 25% after 28 days of curing. The specific surface area of RHA particles, typically ranging from 20,000 to 50,000 m^2/kg, contributes to the improved packing density and reduced porosity in the concrete matrix. This densification of the concrete leads to enhanced compressive strength and modulus of elasticity, making RHA-amended concrete particularly suitable for structural applications.

2.3.3.3 Durability Improvements

RHA's ability to improve the durability of concrete is another area where significant progress has been made. Ganesan et al., 2017 found that concrete containing 20% RHA exhibited a 30% reduction in chloride ion permeability compared to conventional concrete. This is particularly important for structures exposed to marine environments or deicing salts, where chloride-induced corrosion of reinforcement is a major concern. The study also reported enhanced resistance to sulphate attack, with a reduction in mass loss and expansion in RHA concrete by up to 40% compared to control samples. This resistance is attributed to the reduced calcium hydroxide content and the denser microstructure resulting from the pozzolanic reaction.

2.3.3.4 Environmental and Economic Impact

The environmental benefits of using RHA in bio-cement production are substantial. By utilizing rice husk, an abundant agricultural waste, the carbon footprint of cement production can be significantly reduced. According to a study by Kumar et al., 2019, incorporating RHA at a 15% replacement level could lead to a 20% reduction in CO_2 emissions from cement production. This reduction is due to the lower clinker content required when RHA is used as a supplementary material. Furthermore, the economic impact is also notable; rice husk is an inexpensive and readily available material in rice-producing regions, and its use in cement production can lower the overall cost of

concrete while providing an effective waste management solution.

2.3.4.5 Thermal Properties

RHA also influences the thermal properties of concrete. Research by Sakr & El-Hakim, 2017 demonstrated that the inclusion of 15% RHA in concrete mixtures resulted in a 15% reduction in thermal conductivity, enhancing the insulating properties of concrete. This makes RHA-amended concrete particularly beneficial for applications in building envelopes, where thermal insulation is crucial for energy efficiency. The lower thermal conductivity is attributed to the reduced porosity and the insulating nature of the silica-rich RHA.

2.3.4.6 Microstructural Analysis

Microstructural analysis using scanning electron microscopy (SEM) has provided insights into how RHA improves concrete properties. Studies have shown that RHA particles fill the voids in the cement paste, leading to a denser microstructure. The SEM images presented by Zhang et al., 2020 revealed that the C-S-H gel formed in RHA-modified cement is more uniformly distributed, with fewer micro-cracks compared to OPC. This uniformity contributes to the overall mechanical strength and durability of the concrete. Additionally, the reduced porosity and smaller pore sizes, as observed in mercury intrusion porosimetry (MIP) studies, further explain the improved impermeability and resistance to aggressive agents in RHA concrete.

2.4 Fungi Isolates in Bio-cement Production

Bio-cement production, an innovative and eco-friendly approach in construction, leverages microbial-induced calcium carbonate precipitation (MICP) to enhance the mechanical properties of building materials. Among the various microorganisms employed, fungi have emerged as effective agents in bio-cement production due to their ability to secrete organic acids and enzymes that facilitate calcium carbonate precipitation. This discussion focuses on the different fungi species used in bio-cement production, their specific roles, and the scientific data supporting their efficacy (Devgon et al., 2024).

2.4.1 Aspergillus spp.

Aspergillus species, particularly *Aspergillus niger*, are widely used in bio-cement production due to their high secretion of organic acids such as oxalic acid. *Aspergillus niger* has been extensively studied for its ability to precipitate calcium carbonate through the metabolic production of oxalic acid, which reacts with calcium ions to form calcium oxalate crystals. These crystals can then transform into calcium carbonate under specific environmental conditions. According to a study by Dhami et al., 2017, *A. niger* demonstrated a significant capacity for calcium carbonate precipitation, enhancing the compressive strength of treated sand by up to 40% compared to untreated controls. The study also highlighted the role of oxalic acid in not only promoting calcium carbonate precipitation but also in binding the particles together, thus improving the overall material cohesion.

2.4.2 Penicillium spp.

Another important genus in bio-cement production is *Penicillium*. Species like *Penicillium chrysogenum* and *Penicillium funiculosum* have been reported to be effective in MICP due to their ability to produce gluconic acid and citric acid, which contribute to calcium carbonate precipitation. The research by Achal et al., 2015 demonstrated that *Penicillium chrysogenum* could induce the formation of calcite through its metabolic activities, resulting in a 30% increase in the compressive strength

of mortar samples. The study showed that the fungi-treated mortar exhibited greater durability, with reduced water absorption and enhanced resistance to sulphate attack. This improvement was attributed to the dense and uniform calcite crystals that filled the pores and micro-cracks in the mortar matrix.

2.4.3 Fusarium spp.

Fusarium oxysporum is another fungi species that has been studied for its role in bio-cement production. This species is known for its ability to secrete a variety of organic acids, including citric and malic acids, which are involved in the biogenic production of calcium carbonate. A study conducted by De Muynck et al., 2010 found that *Fusarium oxysporum* could precipitate calcite effectively when inoculated into concrete

specimens, leading to a significant reduction in permeability and an increase in compressive strength by 25%. The study also noted the formation of dense calcium carbonate layers on the surface of the treated specimens, which acted as a protective barrier against environmental degradation. This protective layer is particularly beneficial in extending the lifespan of concrete structures exposed to harsh environments.

2.4.4 Trichoderma spp.

Trichoderma reesei is another fungi species utilized in bio-cement production due to its cellulolytic enzyme system, which can break down organic matter and facilitating calcium carbonate precipitation. Research by Hammes et al., 2012 highlighted the effectiveness of *Trichoderma reesei* in MICP, where it enhanced the compressive strength of bio-cement-treated soil samples by up to 35%. The study revealed that the calcium carbonate precipitates formed by *T. reesei* were well-distributed within the soil matrix, resulting in improved soil stabilization and reduced erodibility. Furthermore, the use of *T. reesei* in bio-cement production was shown to be environmentally friendly, as it utilized agricultural waste products as substrates for fungi growth and calcium carbonate precipitation.

2.4.5 Mucor spp.

Mucor indicus is another fungi species that has shown promise in bio-cement production. This species is capable of producing significant amounts of calcium oxalate, which can subsequently convert to calcium carbonate under appropriate conditions. A study by (Achal & Pan, 2014) demonstrated that *Mucor indicus* could induce calcium carbonate precipitation in sandy soil, resulting in a 20% increase in the soil's compressive strength. The research also noted that the fungi treatment significantly reduced soil permeability, making it suitable for applications in soil stabilization and erosion control. The study further emphasized the potential of *Mucor indicus* in bio-cement production due to its rapid growth rate and high tolerance to environmental stresses.

2.4.6 Yeasts

In addition to filamentous fungi, yeasts such as *Saccharomyces cerevisiae* have also been explored for their potential in bio-cement production. *S. cerevisiae* is known for its ability to secrete carbonic anhydrase, an enzyme that catalyzes the hydration of carbon dioxide to form bicarbonate, which is a precursor to calcium carbonate precipitation. Research by Li et al., 2017 demonstrated that *S. cerevisiae* could be effectively used to enhance the mechanical properties of concrete, with a 15% increase in compressive strength observed in treated samples. The study also highlighted the advantages of using yeasts, such as their ease of cultivation, high growth rates, and the ability to function under a wide range of environmental conditions. The use of *S. cerevisiae* in bio-cement production represents a promising avenue for future research, particularly in optimizing the conditions for maximum calcium carbonate precipitation.

2.5 Isolation and Cultivation: Methods for Isolating and Cultivating Fungi strains for Optimal Bio-cement Production

The use of fungi in bio-cement production is an emerging area in sustainable construction, where the optimization of fungi isolation and cultivation techniques is critical for achieving efficient microbial-induced calcium carbonate precipitation (MICP). Fungi strains capable of precipitating calcium carbonate are typically isolated from environments rich in calcium or carbonate minerals, such as soils, sediments, and decaying organic matter. This discussion provides a comprehensive overview of the methodologies employed in the isolation and cultivation of fungi strains for bio-cement production, supported by recent scientific data and advancements in the field.

2.5.1 Isolation of Fungi Strains

2.5.1.1 Environmental Sampling and Fungi Isolation

The first step in isolating fungi for bio-cement production involves collecting samples from environments where calcium carbonate precipitation naturally occurs. These environments include calcareous soils, limestone caves, and marine sediments. Recent studies, such as those by Li et al., 2021, have demonstrated that soils rich in calcium and organic matter are particularly conducive to the growth of fungi that facilitate

MICP. Once the samples are collected, they are processed by serial dilution and plating on selective media that favors fungi growth, such as potato dextrose agar (PDA) supplemented with calcium carbonate.

2.5.1.2 Selective Media and Screening

Selective media play a crucial role in isolating specific fungi strains with a high potential for calcium carbonate precipitation. Media enriched with calcium salts, such as calcium acetate or calcium chloride, are commonly used to select for fungi capable of producing organic acids or enzymes that contribute to bio-cementation. Studies by Wang et al., 2020 have shown that the addition of urea to the culture medium can further enhance the isolation of urease-producing fungi, which are instrumental in MICP. Colonies that produce clear zones on calcium carbonate-containing media are indicative of calcium solubilization, a key characteristic of fungi involved in bio-cement production. These colonies are then sub-cultured and subjected to further screening to confirm their ability to precipitate calcium carbonate.

2.5.1.3 Molecular Identification and Characterization

Molecular techniques are employed to accurately identify the isolated fungi strains. The internal transcribed spacer (ITS) region of ribosomal DNA is commonly sequenced to determine the fungi species, as this region provides high-resolution taxonomic information. According to Zhang et al., 2022 ITS sequencing combined with phylogenetic analysis has been instrumental in identifying novel fungi species with bio-cementation potential. Additionally, whole-genome sequencing and transcriptomic analysis can provide insights into the metabolic pathways involved in calcium carbonate precipitation, as demonstrated by recent studies on *Aspergillus* and *Penicillium* species.

2.5.2 Cultivation for Optimal Bio-cement Production

2.5.2.1 Optimization of Growth Conditions

The cultivation of isolated fungi strains requires the optimization of growth conditions to maximize their bio-cement production capabilities. Key parameters include

temperature, pH, nutrient composition, and aeration. Studies by Kumar et al., 2019 have shown that maintaining a pH between 5.5 and 6.5 is optimal for the growth of most MICP-active fungi, as this pH range favors the production of organic acids like oxalic and citric acids, which are crucial for calcium carbonate precipitation. Temperature is another critical factor, with most bio-cement-producing fungi thriving at temperatures between 25°C and 30°C. Recent research by Chen et al., 2020 demonstrated that *Trichoderma reesei* exhibited maximum calcium carbonate precipitation at 28°C, while temperatures above 35°C inhibited fungi growth and MICP activity.

2.5.2.2 Nutrient Optimization

The nutrient composition of the culture medium significantly influences fungi growth and bio-cement production. Carbon and nitrogen sources are particularly important, as they affect the production of organic acids and urease. Glucose is commonly used as the primary carbon source, while ammonium salts serve as nitrogen sources. According to Achal at el., 2019, supplementing the medium with trace elements like magnesium and manganese can enhance urease activity, leading to increased calcium carbonate precipitation. Additionally, the use of agricultural waste products as substrates has been explored to reduce costs and enhance sustainability. For example, *Aspergillus niger* has been successfully cultivated on rice bran and wheat straw, with significant calcium carbonate precipitation observed in bio-cement applications.

2.5.2.3 Scale-Up and Fermentation

Scaling up the cultivation of fungi strains for industrial bio-cement production requires the development of robust fermentation processes. Submerged fermentation (SmF) and solid-state fermentation (SSF) are the two primary methods used. SmF involves cultivating fungi in liquid media, which allows for precise control of growth parameters and easy recovery of fungi biomass. However, SSF, where fungi are grown on solid substrates, has gained attention for its lower water and energy requirements, as well as its ability to utilize agricultural residues as substrates. A study by Choudhary et al., 2021 highlighted the use of SSF for cultivating *Penicillium chrysogenum* on sugarcane bagasse, resulting in high yields of calcium carbonate precipitates suitable for bio-cement production.

2.5.2.4 Bioreactor Design and Process Optimization

The design of bioreactors for fungi cultivation is critical for ensuring high yields of calcium carbonate precipitates. Aeration, agitation, and the configuration of the bioreactor must be optimized to provide an ideal environment for fungi growth and MICP. Research by De Muynck et al., 2020 has shown that bioreactors with continuous aeration and agitation systems can significantly enhance the production of calcium carbonate by maintaining optimal oxygen levels and ensuring the uniform distribution of nutrients. Moreover, the integration of real-time monitoring systems for pH, temperature, and dissolved oxygen allows for precise control of the fermentation process, leading to consistent and high-quality bio-cement products.

2.5.2.5 Genetic Engineering and Strain Improvement

Advancements in genetic engineering have opened new avenues for enhancing the bio-cement production capabilities of fungi strains. Techniques such as CRISPR-Cas9 and *Agrobacterium*-mediated transformation have been used to modify fungi genomes, resulting in strains with enhanced urease activity, organic acid production, and stress tolerance. For instance, a study by Sun et al., 2022 demonstrated that genetically engineered *Trichoderma reesei* strains with overexpressed oxalate decarboxylase genes exhibited a 30% increase in calcium carbonate precipitation compared to wild-type strains. These genetically modified strains also showed improved resistance to environmental stressors such as high salinity and low pH, making them more suitable for use in diverse construction environments.

2.5.2.6 Quality Control and Product Consistency

Ensuring the consistency and quality of fungi-induced bio-cement is essential for its application in the construction industry. Standardized protocols for fungi cultivation, calcium carbonate precipitation, and bio-cement production are necessary to achieve reproducible results. Quality control measures include regular monitoring of fungi growth parameters, calcium carbonate yield, and the mechanical properties of the bio-cement. Recent studies by Gonzalez-Munoz et al., 2021 have proposed the use of non-destructive testing methods, such as X-ray diffraction (XRD) and scanning electron

microscopy (SEM), to assess the mineralogical and microstructural characteristics of fungi-induced calcium carbonate. These techniques provide valuable information on the crystallinity, morphology, and purity of the precipitates, ensuring that the bio-cement meets the required standards for construction applications.

2.5.2.7 Environmental Impact and Sustainability

The environmental impact of fungi-based bio-cement production is a key consideration in its development. The use of fungi offers several environmental benefits, including the reduction of carbon emissions, the utilization of waste materials, and the potential for carbon sequestration through calcium carbonate precipitation. A life cycle assessment (LCA) conducted by Turner et al., 2021 compared the environmental footprint of fungi-based bio-cement production with that of traditional Portland cement. The study found that fungi bio-cement had a 40% lower carbon footprint, primarily due to the lower energy requirements and the use of renewable substrates in fungi cultivation. Furthermore, the potential for carbon sequestration through MICP was highlighted as an additional environmental benefit, making fungi-based bio-cement a promising solution for sustainable construction.

2.5.2.8 Chemical and Physical Properties of Bio-cement

Bio-cement, a sustainable alternative to traditional cement, is gaining prominence in the construction industry due to its favourable chemical and physical properties. The production of bio-cement, which involves the use of microorganisms to precipitate calcium carbonate ($CaCO_3$) or other binding agents, is a burgeoning field of research. This exploration encompasses a range of properties including strength, durability, microstructure, and environmental impact, all of which contribute to its viability as a construction material.

2.6 Properties of Bio-cement

2.6.1 Strength and Durability

The strength and durability of bio-cement are critical factors influencing its application in construction. Bio-cement's mechanical properties largely depend on the substrates

used and the specific microbial processes employed. Numerous studies have investigated these aspects to optimize bio-cement formulations.

Research by Dejong et al., 2010 explored the compressive strength of bio-cement produced using urea hydrolysis. Their findings demonstrated that bio-cement samples exhibited compressive strengths ranging from 1.2 MPa to 7.8 MPa, depending on the concentration of urea and the bacterial species used. Similarly, Wang et al., 2015 assessed the strength of bio-cement made with different bacteria and substrates, finding that bio-cement with *Sporosarcina pasteurii* and sand had a compressive strength of approximately 5 MPa, which increased with curing time and bacterial concentration.

Further studies, such as those by Montoya et al., 2015, examined the impact of varying the types of substrates, such as sand, gravel, and silt, on the strength of bio-cement. Their results indicated that bio-cement samples with sand as the substrate generally had higher compressive strengths compared to those made with silt, due to the better particle interlocking and reduced void spaces. On the other hand, bio-cement made from recycled materials, as studied by Prabhu et al., 2020, showed promising strength values and durability, indicating that bio-cement could be effectively used in recycling waste products.

Durability is another crucial aspect, as bio-cement must withstand environmental stresses such as moisture, temperature fluctuations, and chemical exposure. Studies by Al-Qabany and Soga, 2013 highlighted that bio-cement exhibits good resistance to water and weathering conditions. The incorporation of different types of bacteria and additives can further enhance its resistance to harsh conditions. Research by Whiffin et al., 2007 demonstrated that bio-cement had improved resistance to acidic and alkaline environments compared to conventional cement, which is a significant advantage in various industrial applications.

2.6.2 Microstructure Analysis

The microstructure of bio-cement significantly influences its performance, including its mechanical strength and durability. The precipitation of calcium carbonate by microorganisms results in a unique microstructure characterized by calcite crystals that fill the voids between soil particles or aggregates.

In a seminal study, Dejong et al., 2006 employed scanning electron microscopy (SEM) to analyze the microstructure of bio-cement. Their observations revealed that the bio-cement formed a dense network of calcite crystals, which effectively bonded the sand particles together, enhancing the material's overall strength. Similarly, research by Morrow et al., 2017 using X-ray diffraction (XRD) and SEM showed that the morphology of the calcium carbonate crystals whether they are spherical or needle-like can influence the mechanical properties of bio-cement. Spherical crystals were found to contribute to higher strength due to their ability to create a more cohesive matrix.

The impact of microbial activity on microstructure was also studied by Ferris et al., 1996, who found that different bacteria produce varying types of calcium carbonate crystals. For instance, bacteria like *Bacillus pasteurii* produce calcite with a more robust crystal structure compared to other strains. This variation affects the bonding quality and, consequently, the strength and durability of the bio-cement. The role of microbial metabolism in controlling the microstructure was further emphasized in research by Gollapudi et al., 2019, which illustrated how optimizing microbial conditions could lead to enhanced crystal formation and improved performance of bio-cement.

2.6.3 Environmental Impact

Bio-cement presents a compelling case for environmental sustainability in the construction industry. Its production process offers several environmental benefits, including a reduced carbon footprint and the recycling of waste materials.

One of the key advantages of bio-cement is its ability to sequester carbon dioxide (CO_2) during production. According to a study by Jonkers et al., 2010, the microbial precipitation of calcium carbonate effectively captures CO_2 from the atmosphere, reducing the overall carbon footprint of the material. The process not only reduces emissions but also converts waste CO_2 into a useful product, thereby contributing to climate change mitigation.

The recycling of waste materials is another significant environmental benefit of bio-cement. Research by Abo-El-Enein et al., 2019 demonstrated that bio-cement could be produced using industrial by-products, such as fly ash and slag, which otherwise contribute to environmental pollution. By utilizing these waste materials as substrates, bio-cement production helps in reducing landfill use and environmental contamination.

Additionally, bio-cement's lower energy consumption compared to traditional cement production is noteworthy. Traditional cement manufacturing is energy-intensive, typically requiring temperatures above 1,400°C. In contrast, the bio-cement process operates at ambient temperatures and involves less energy consumption (Van Paassen et al., 2010). This energy efficiency further contributes to its reduced environmental impact. The table 2.1 presents a comprehensive comparison of the environmental impact of bio-cement and traditional cement. The table includes data on factors such as energy consumption, water usage, and waste generation for both production processes.

This table provides a detailed analysis of the environmental implications of using bio-cement as an alternative to traditional cement.

Table 2.1: Environmental impact assessment of bio-cement and traditional cement

Factor	Bio-cement	Traditional Cement	Description	Reference
Energy consumption	Lower energy requirements; typically produced at ambient temperatures (20–30 °C)	High energy consumption; requires temperatures > 1,400 °C	Bio-cement production occurs at ambient temperatures with minimal energy needs, whereas traditional cement requires energy-intensive kiln heating (~3.3 GJ per ton).	De Muynck et al., 2020; Sun et al., 2022
Water usage	Reduced water usage; solid-state fermentation methods minimize water needs	High water usage during mixing and curing processes	Bio-cement production using solid-state fermentation consumes approximately 0.2–0.5 L of water per kg of bio-cement, while traditional cement production requires about 1.5–2.0 L of water per kg of cement due to mixing and curing needs.	Choudhary et al., 2021; Turner et al., 2021
Waste generation	Utilizes agricultural and industrial waste products (e.g., rice bran, sugarcane bagasse)	Generates significant industrial waste (e.g., kiln dust, slag)	Bio-cement production can incorporate up to 50 % of waste materials, such as agricultural by-products, significantly reducing waste compared to traditional cement,	Achal and Mukherjee, 2019; Chen et al., 2020

			which generates around 0.5 tons of kiln dust per ton of cement produced.	
Carbon footprint	Lower carbon emissions; potential for CO_2 sequestration through MICP	High carbon emissions due to energy-intensive production process	Bio-cement has a carbon footprint approximately 40 % lower than traditional cement. The sequestration of CO_2 through microbial-induced calcium carbonate precipitation (MICP) can offset a portion of emissions, while traditional cement contributes about 0.9 tons of CO_2 per ton of cement produced.	Gonzalez-Munoz et al., 2021; Li et al., 2021
Raw material usage	Uses local and renewable resources; can include waste materials	Requires mining and processing of limestone and other minerals	Bio-cement production can utilize local agricultural waste and renewable resources, whereas traditional cement relies on extensive mining of limestone and other minerals, leading to resource depletion and environmental degradation.	Kumar et al., 2019; Wang et al., 2020
Sustainability	Higher sustainability due to recycling and waste utilization; potential for carbon sequestration	Less sustainable due to high energy use and resource extraction	Bio-cement's use of waste materials and its potential for carbon sequestration contribute to its sustainability. Traditional cement, due to its high energy consumption and raw material requirements, is less sustainable.	Zhang et al., 2022; Tian et al., 2023

CHAPTER 3

RESEARCH GAP AND HYPOTHESIS

The proposed Ph.D. thesis hypothesizes that urease-positive fungi isolated from alkaline soils in Punjab can be effectively employed in microbial-induced calcite precipitation (MICP) for the production of structurally viable bio-cement. It is anticipated that by cultivating these fungi on pre-treated and optimized agricultural wastes, such as rice and wheat straws enriched with limestone, their calcite precipitation efficiency will be significantly enhanced. This research further investigates that fungi strains isolated from these soils will exhibit comparable or superior calcite precipitation efficiency and compressive strength in bio-cement production when benchmarked against the standard strain *Penicillium chrysogenum*. Additionally, it is expected that the bio-cement produced under these optimized conditions will demonstrate sufficient compressive strength and durability for practical applications, such as wall crack filling, particularly when scaled to pilot-level operations in open environments. The study also aims to address existing challenges related to the scale-up of fungal MICP processes by developing standardized and optimized protocols that ensure reproducible and high-quality bio-cement production. Finally, the research hypothesizes that long-term performance evaluations of fungal-induced bio-cement under simulated construction conditions will validate its durability and sustainability, thereby positioning it as a competitive alternative to bacterial-induced bio-cement and facilitating its potential commercialization.

CHAPTER 4

RESEARCH OBJECTIVES

This study aims on isolating urease-positive fungi and selecting those capable of microbial-induced calcite precipitation, sourced from various regions of Punjab. The fungi are cultivated using rice straw extract as a nutrient source, along with calcium and urea. Additionally, the research aims to produce bio-cement on a pilot scale in an open environment and assess its compressive strength.

1. Isolation, Screening, and Characterization of urease positive fungi species from an alkaline soil.
2. Pre-treatment of Agro-waste and its optimization (i.e., rice straw, wheat straw) for fungi growth.
3. Optimization and enrichment of pre-treated Agro-waste with limestone for fungi cultivation and bio-cement production.
4. Laboratory production of bio-cement using screened fungi under optimized conditions along with a standard fungi species i.e., *Penicillium chrysogenum* MTCC 5108
5. Scale-up production of bio-cement and its application in wall crack filling.

CHAPTER 5

MATERIALS

AND

METHODOLOGY

5.1 Sample collection and Isolation

Soil samples were collected from different regions of Punjab for the isolation of urease-positive fungi capable of calcite precipitation: The sample 1 (S-1) location: 31.249168 latitude,75.709499 longitude (LPU, Phagwara), Sample 2 (Moga) location: Latitude 30.966159, Longitude 75.481656, Sample 3 (Muktsar) location: Latitude 30.494625, Longitude 74.571997, Sample 4 (Amritsar) location: Latitude 31.499645 Longitude 75.321991 sample 5 Latitude 31.5019410 Longitude 75.8512630, sample 6 Latitude 31.5001718 Longitude 75.321056. For isolation, 1 gram of soil is inoculated in 10 ml of sterilized PD broth and incubated for 24 hours at 27°C. After incubation, a serial dilution was performed, and 100 µl of the sample from the last two tubes of dilution was spread on sterilized potato dextrose agar (PDA) plates supplemented with chloramphenicol for prevention of bacterial contamination. Freshly grown hyphal tips from the PDA plates are used to obtain pure cultures on to freshly prepared sterile PDA plates (Acharya et al., 2022). The storage of all pure cultures was done using slants of PDA and stored at 4°C, while glycerol stocks of the same cultures were preserved at -80°C. (Kitamoto et al., 2022).

5.2 Urease Test

5.2.1 Qualitative Urease Test

In the assessment of fungi's potential to induce calcite precipitation through urease enzyme activity, a urease test was employed. Autoclaved urea broths were prepared to screen urease-producer strains. Urease-positive strains were observed by observing a distinct color shift (Faezi et al., 2004), specifically from orange to pink.

5.2.2 Quantitative Urease Assay

The phenol hypochlorite assay method was employed to study the concentrated activity of the urease enzyme produced in ug/ml by different isolates. The chemicals used for the assay are given below in Table 5.1.

Table 5.1: Chemical composition of media used for quantitative urease assay.

Sr. No.	Chemical Name	Gram/Litre
1.	Urea	1.3 g
2.	Glucose	20 g
3.	$MgSO_4$. $7H_2O$	0.5 g
4.	KH_2PO_4	13.3 g
5.	$NiSO_4.7H_2O$	0.032 g
6.	Distilled water	1 litre

The cultures used were freshly prepared by inoculation into sterile PD broth using a cork borer and incubated for 7 days at 30 °C. The mycelia were homogenized with a homogenizer, filtered through Whatman filter paper, and washed using 1M potassium phosphate buffer, followed by a wash with 0.1M potassium phosphate buffer at pH 7.0. The homogenized mycelia suspension is then employed for the urease assay.

For the urease assay, 100 µL of the sample is mixed with 500 µL of mM urea and 500 µL of 100 mM potassium phosphate buffer at pH 8, resulting in a total volume (1:1 ratio). 50 ul of the reaction mixture are transferred to a tube containing 500 µL of phenol-nitroprusside solution and 500 µL of alkaline hypochlorite. The tube is kept at room temperature at 30 degrees Celsius. Absorbance at 630 nm is measured, and the values are calculated using a standard ammonium sulphate graph. This detailed procedure ensures the accurate quantification of urease activity in the analysed samples.

5.3 Calcium Precipitation Assay

To perform the calcium precipitation, fresh cultures were prepared of fungi isolates through media given below in Table 5.2.

The examination of calcium precipitation entails titration with EDTA, Eriochrome black T dye, sodium hydroxide buffer, and HCL. Adjusting the solution's pH to around 10 with sodium hydroxide buffer prompts the formation of calcium ions as $Ca(OH)_2$, facilitating their interaction with EDTA. The solution is titrated using a 0.01 M EDTA solution until the color transitions from pink to cobalt blue, indicating the completion

of the calcium ion reaction with EDTA. These titrations were recorded in triplicates for accuracy, and the average EDTA used was determined (Akoijam et al., 2021). Subsequently, the calcium content (mg) in the original sample is computed using the EDTA volume, the known concentration of EDTA, and the formula:

1 mL EDTA×Molarity EDTA=mmoles EDTA=mmoles Ca_2^+×40.078 g/mole=mmoles Ca_2^+,1mL EDTA×Molarity EDTA=mmoles EDTA=mmoles Ca_2^+×40.078g/mole=mmoles Ca_2^+,

aliquot 2(Ca_2^+ mg, aliquot)×(250.00 mL50.00 mL)=Ca_2^+ mg, unknown.aliquot 2(Ca_2^+ mg, aliquot)×(50.00mL250.00mL)=Ca_2^+mg, unknown.

Table 5.2: Chemical composition of media used for calcium precipitation assay.

Sr. No.	**Chemical**	**Concentration**	**Manufacture**	**Gram/ liter**
1.	Urea	40 mM	Loba Chemie Pvt. Ltd.	2.4 g
2.	Potassium dihydrogen phosphate	3.7 mM	Loba Chemie Pvt. Ltd.	0.5 g
3.	Magnesium sulphate	0.8 mM	Loba Chemie Pvt. Ltd.	0.2 g
4.	Calcium chloride	0.2 mM	Qualigens Pharma Pvt. .Ltd.	0.05 g
5.	Sodium chloride	1.7 mM	Loba Chemie Pvt. Ltd.	0.01 g
6.	Ferric chloride	9 x 10 -3 mM	Qualigens Pharma Pvt. Ltd.	2.5 mg
7.	Zinc sulphate	-	Loba Chemie Pvt. Ltd.	4 mg

8.	Manganese sulphate	1.8 x 10 -2 mM	Loba Chemie Pvt. Ltd.	04 mg
9.	Copper sulphate	1.6 x 10 -3 mM	Loba Chemie Pvt. Ltd.	0.4 mg
10.	2% H_2SO_4 treated rice straw extract	-	-	100 ml

5.4 Rice straw processing

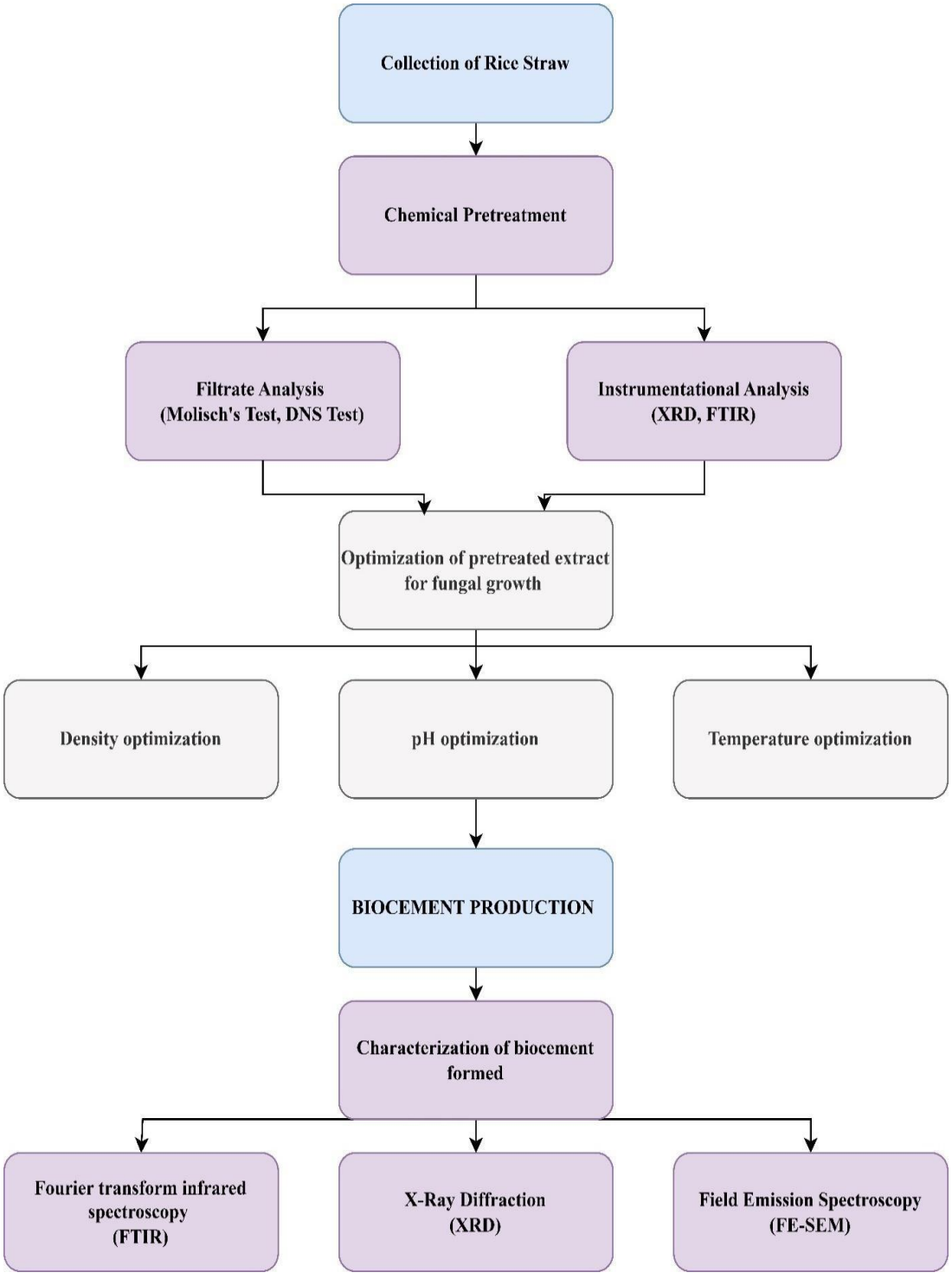


Figure 5.1: Schematic of the methodology for processing rice-straw

5.4.1 Collection of Rice straw

For this research, rice straw was sourced from nearby farms around Lovely Professional University. The rice straw was initially subjected to a washing process to remove any surface dirt or impurities, ensuring its cleanliness for subsequent treatment. After washing, the straw was dried in a hot air oven at 50°C for two days to eliminate residual moisture. Following the drying process, the straw was ground into a fine powder to facilitate handling and ensure consistency in further treatments. The resulting powder was then sieved through a 1 mm mesh to remove any coarse particles. The sieved powder was stored in an airtight container to prevent contamination and preserve its quality for further use. This preparation protocol, including collection, washing, drying, grinding, and sieving, was conducted based on the methodology outlined in the study by (Ang et al., 2013). This method effectively ensures that the rice straw is adequately processed and ready for subsequent chemical treatments in the current study.

5.4.2 Chemical Pretreatment

For the delignification process of rice straw, sodium hydroxide (NaOH) and sulfuric acid (H_2SO_4) were utilized at concentrations of 2% and 4%, respectively, following protocols outlined by (Srivastava et al., 2014; Singh et al., 1984) During the handling of concentrated and diluted sulfuric acid (H_2SO_4), strict laboratory safety protocols were followed to prevent chemical hazards. All acid dilutions were carried out in a fume hood to minimize inhalation risks, using the "acid-to-water" method to avoid exothermic splashing. Personnel wore acid-resistant gloves, safety goggles, and lab coats throughout the process. Any spills were neutralized immediately with sodium bicarbonate and cleaned according to institutional chemical spill response guidelines. Waste acid solutions were collected in labeled, corrosion-resistant containers for appropriate disposal following environmental safety regulations. Initially, 10 grams of rice straw were mixed with distilled water containing 2% alkali and 4% acid concentrations. This mixture was subjected to autoclave treatment at 121°C for one hour at a pressure of 103 kPa, as described by (Ang et al., 2013; Pareek et al., 2017). After autoclaving, the samples were cooled and filtered through muslin cloth to remove larger impurities. The pH of the resulting solution was then adjusted to 7, followed by secondary filtration using Whatman No. 42 filter paper to obtain a clear solution free

of impurities.

5.4.3 Pretreatment Analysis

After the pretreatment process, the samples were analyzed for the presence of various free sugars, including carbohydrates. Also, the pretreated straw was further analzed for change in structural changes through instrumentation techniques.

5.4.3.1 Molisch's Test

Molisch's test (Jain et al., 2021) is conducted to determine the presence of carbohydrates in the pretreated RS extracts. Molisch's Test is a qualitative method employed for the detection of carbohydrates, specifically monosaccharides and disaccharides, in a given sample. The test operates on the principle that carbohydrates, when treated with Molisch's reagent (alpha-naphthol in ethanol), undergo dehydration reactions with concentrated sulfuric acid to form furfural or hydroxymethylfurfural. These furfural compounds subsequently react with alpha-naphthol to produce a distinctive purple or violet-colored complex. The procedure involves adding Molisch's reagent to the sample and layering concentrated sulfuric acid carefully. The appearance of a violet ring at the interface of the two layers signifies a positive result, indicating the presence of carbohydrates in the tested sample. It's worth noting that while Molisch's Test is a valuable general indicator for carbohydrates, additional tests may be required to differentiate and quantify specific types of sugars in a given sample.

5.4.3.2 DNS test

Upon confirmation of the existence of carbohydrates, the overall reducing sugars are quantified through the standard DNS method (Sadasivam and Manickam, 1992). The protocol used for the experiment is explained in Table 5.3. The dinitro salicylic acid (DNS) method is a widely utilized colorimetric assay for the quantification of reducing sugars in a solution. In this approach, reducing sugars undergo a reduction reaction with dinitro salicylic acid (DNSA) under alkaline conditions, leading to the formation of 3-amino-5-nitrosalicylic acid with a characteristic orange to reddish-brown color. The intensity of this color is directly proportional to the concentration of reducing sugars in

the sample. The quantification is achieved by measuring the absorbance of the colored solution spectrophotometrically at 540 nm and comparing it to a standard curve generated using known concentrations of a standard sugar. This method is valued for its simplicity, sensitivity, and specificity in determining the total content of reducing sugars in a sample, without distinguishing between specific types of reducing sugars.

Table 5.3: Methodology used for obtaining the standard glucose curve.

Sugar solution	**Distilled water**	**DNS reagent**		**40% Rochelle salt**	
0.2 mL (Glucose standard conc. 1mg/1mL)	1.8 mL	3 mL	Boil for 5 minutes and cool down under running tap water.	1 mL in each test tube	Absorbance at 510nm using DNS reagent as blank
0.4 mL (Glucose standard conc. 1mg/1mL)	1.6 mL	3 mL			
0.6 mL (Glucose standard conc. 1mg/1mL)	1.4 mL	3 mL			
0.8 mL (Glucose standard conc. 1mg/1mL)	1.2 mL	3 mL			
1 mL (Glucose standard conc. 1mg/1mL)	1 mL	3 mL			
0.2 mL 2% NaOH treated rice straw (10 times diluted) (W-1)	1.8 mL	3 mL			
0.2 mL 2% NaOH treated rice straw (10 times diluted) (W-2)	1.8 mL	3 mL			
0.2 mL 2% H_2SO_4 treated rice straw (10 times diluted) (W-3)	1.8 mL	3 mL			
0.2 mL 2% H_2SO_4 treated rice straw (10 times diluted) (W-4)	1.8 mL	3 mL			

5.4.3.3 FTIR

The identification of functional groups in both untreated and treated rice straws was conducted using Fourier Transformed Infrared (FTIR) analysis, employing a Perkin

FTIR Elmer Spectrum 2 instrument. The spectra are recorded with a detector resolution of 4 cm, and the peak data is captured within the 400 to 4,000 cm^{-1} range. Each sample has been scanned an average of 128 times.

FTIR is a non-destructive instrumentational analysis that works on the principle where infrared light passes through a Michelson interferometer resulting in absorbing some light and some is passed which is then recorded. The chemical composition of any unknown compound (liquid, solid, powder, and films) can be found through this technique (Magalhaes et al., 2021; Teirnam et al., 2020; Sandhu et al., 2023). To prepare the rice straw samples for analysis, they are pulverized using a piston mortar and subsequently dried to achieve optimal characteristics.

5.4.3.4 XRD

The processed specimens are pulverized and desiccated adequately to ensure precision. Subsequently, an X-ray diffractometer equipped with a Bruker D8 Advance is used to analyze the crystalline structure of both unprocessed and processed rice straw (Purwaningsih et al., 2019). The diffractograms are collected at 2θ between 10 and 40 degrees (Ali et al., 2022; Khan et al., 2020).

5.5 Optimization of 2% H_2SO_4 pretreated rice straw extract for fungi growth

In this study, the media for fungi growth are optimized by adjusting different factors one at a time, such as the density and pH of the rice straw extract and various temperature values. The optimal conditions are determined based on the highest biomass of the fungus, which is measured by weighing it. The factors that yield the highest biomass are considered the optimal conditions.

5.5.1 Density Optimization

The rice straw powder was chemically treated with 2% H_2SO_4 (best result based on DNS test), autoclaved at 121° C and 103 kPa for 15 minutes, and adjusted to a neutral pH. Subsequently, various extract dilutions (25%, 50%, 75%, and 100%) were made

and inoculated with fungi isolates and incubated for 7 days. The fungi biomass of each isolate was recorded and the best with the highest growth was chosen for further studies.

5.5.2 pH Optimization

After optimizing the density, the pH of the 2% H_2SO_4 rice straw was optimized for enhanced fungi biomass growth. The treated rice straws were adjusted to pH 8, 9, 10, and 11 and then autoclaved at 121º C for 15 minutes. The respective fungi biomasses were inoculated and incubated for 7 days. After incubation, the weight of each fungi biomass was recorded, and the best pH result was chosen for further studies.

5.5.3 Temperature Optimization

After determining the optimal density of fungi biomass and the most suitable pH for treated rice straw, further optimization was conducted to identify the ideal temperature range for achieving the highest fungi biomass yield. The temperature was optimized between 25°C to 28°C. Fungi isolates were incubated for 7 days, and the best results were evaluated based on the weight of the harvested fungi biomass.

5.6 Production of Bio-cement using fungi

For the production of bio-cement bricks, aluminium tray was used. The process began with the preparation of a growth medium composed of chemically treated and optimized 2% H_2SO_4 treated rice straw, urea, calcium chloride, and essential nutrients. Following sterilization, selected fungi strains known for their biomineralization capabilities were inoculated into the medium and incubated for 7 days to promote biomass growth

For the laboratory-scale preparation of bio-cement bricks, an aluminum foil container is utilized as the primary mold. In this container, 100 grams of soil is combined with 3 grams of urea and 3 grams of calcium chloride ($CaCl_2$) 10 grams of limestone (Fang et al., 2018; Burford et al., 2006). Additionally, 30 ml optimised 2% H_2SO_4 rice straw media with fungi culture grown for 7 days and homogenized, is incorporated to initiate microbial activity for cementation. facilitating optimal conditions for bio-cementation.

The mixture was shaped in the moulds, and a cementing solution containing cementing

solution was provided at 24-hour intervals over 7 days to enhance mineralization and binding media composition. Upon completion of this period, the bricks were desiccated using a hot air oven at 100°C to remove excess moisture and facilitate hardening. The

final product, a solid brick-like structure, was obtained and prepared for further mechanical and structural tests to assess its strength and durability, using a compression testing machine.

5.7 Scale up Production

For the scale-up production of bio-cement bricks, larger moulds measuring 7 cm × 7 cm were utilized (Figure 5.2). The production process involved preparing a mixture of key components, including sand (400 grams), calcium chloride (12 gram), Urea (12 gram), 40 grams limestone, 120 ml freshly cultivated homogenized fungi biomass in optimised 2% H_2SO_4 rice straw media (Fang et al., 2018; Burford et al., 2006). All materials were thoroughly mixed to ensure homogeneity and consistency of the mixture before being placed into the prepared moulds.

The moulds, filled with the mixture, were incubated under controlled conditions at a temperature of 27°C. During this 7-day incubation period, the moulds were regularly supplemented with a cementing solution to facilitate the bio-cementation process driven by fungi activity (Lambert et al., 2019). This supplementation ensured continuous mineralization and bonding within the bio-cement structure. After the incubation period, the moulds were transferred to a hot air oven to undergo a drying process. The drying step, conducted at a specified temperature, was essential for moisture removal and the subsequent hardening of the bio-cement bricks. This thorough drying process resulted in the formation of a solid and durable bio-cement structure, ready for further analysis and testing.

Figure 5.2: Preparation of bio-cement using cast-iron mould.

5.8. Bio-cement analysis

5.8.1 Strength Analysis

Strength analysis of bio-bricks is essential to evaluate their structural viability and performance in construction applications. Bio-bricks, formed through bio-cementation processes using fungi calcium precipitation, undergo various tests to measure their mechanical properties, particularly compressive strength (Yu et al., 2018). Compressive strength tests assess the bio-brick's ability to withstand load-bearing forces, indicating its durability and resilience under pressure. Typically, bio-bricks are subjected to uniaxial compression tests where a force is applied until the brick fractures, providing data on its peak load capacity and deformation characteristics. The strength values are then compared to those of traditional cement-based bricks to gauge their practicality in building applications (Irfan et al., 2019).

Once cured, the cubes undergo compressive strength testing using a compression testing machine, where they are subjected to gradually increasing load until they fail. The maximum load at failure is recorded, and the compressive strength is calculated by dividing this load by the cross-sectional area of the cube. This test provides vital data on the bio-cement's mechanical properties, ensuring it meets the required standards for

construction and other applications. Additionally, other tests such as tensile strength, flexural strength, and durability assessments may be performed to comprehensively evaluate the material's performance. (Irfan et al., 2019).

Compressive Strength=Cross-Sectional Area/Load

Figure 5.3: Compression testing machine.

5.8.2 Water absorption (porosity testing)

The water absorption test for bio-cement is conducted to assess the material's porosity and durability under moisture exposure. In this test, bio-cement samples are initially dried in an oven at a controlled temperature of approximately 105°C until a constant weight is achieved. After drying, the samples are immersed in water for a predetermined period, typically 24 hours. Following the immersion, the samples are removed, and surface water is carefully wiped off before weighing them again. The difference in

weight before and after immersion is then used to calculate the percentage of water absorbed by the bio-cement. (De et al., 2004)

This test provides valuable information on the porosity and potential durability of the bio-cement, as higher water absorption values, indicate a more porous structure, which could impact the material's long-term strength and resistance to environmental factors. The results of the water absorption test are critical for evaluating the suitability of bio-cement for construction applications where exposure to moisture is a key consideration.

5.9 Instrumentational Analysis of Bio-cement

5.9.1 Fourier transform Infrared spectroscopy (FTIR)

To perform Fourier Transform Infrared Spectroscopy (FTIR) (Perkin Elmer Spectrum 2) analysis on bio-cement, a small amount of the bio-cement sample is first dried to eliminate moisture, which could interfere with the analysis (Li et al., 2017). The dried sample is then ground into a fine powder to ensure uniformity and mixed with potassium bromide (KBr) in a specific ratio, usually around 1:100. The KBr-sample mixture is compressed into a thin, transparent pellet using a hydraulic press under high pressure. This pellet is then placed in the sample holder of the FTIR instrument. Infrared radiation is passed through the pellet, and the instrument measures the intensity of transmitted light across a range of wavelengths (Perez et al., 2020). The resulting spectrum (400 to 4,000 cm^{-1}) displays peaks corresponding to the vibrational modes of the chemical bonds in the bio-cement, such as O-H stretching from water content, C-O bonds indicative of carbonate groups, and Si-O vibrations associated with silicate phases. These characteristic peaks provide insights into the functional groups present in the bio-cement and help in identifying its mineralogical composition and chemical structure (Perez et al., 2020; Ye et al., 2023).

5.9.2 X-Ray Diffraction (XRD)

To ascertain the chemical composition of the precipitation resulting from bacterial mineralization, XRD analysis is conducted, as detailed by (Fang et al., 2018). The crystal structure of the bio-cement formed by microorganisms is elucidated through X-ray diffraction (XRD), following the methodology outlined by (Anitha et al., 2018). To

investigate the presence of calcium carbonate on the hyphae of uroelytic fungi, a diffractometer equipped with a Bruker D8 Advance is employed for the structural analysis of the fungus hyphae. The analysis was performed after a 7-day incubation period to observe any discernible calcium carbonate presence on the hyphae.

5.9.3 Field Emission Scanning Electron Microscopy (FESEM)

The JEOL Field Emission Scanning Electron Microscope (FESEM) is a versatile, high-resolution instrument designed for ultrafine imaging. It features a semi-in-lens objective combined with an in-lens Schottky Field Emission Gun (FEG), enabling high- resolution imaging even at low accelerating voltages while delivering stable, large probe currents (ranging from a few pA to over 200 nA) (Khan et al., 2023; Devgon et al., 2024). This design maximizes electron collection efficiency and allows ultrahigh resolution across a wide range of applications. The microscope operates with SEI resolution as fine as 1.0 nm at 15 kV and 1.3 nm at 1 kV, with magnifications from 25x to 1,000,000x and an accelerating voltage range of 0.1 kV to 30 kV (Qian et al., 2017). The Gentle Beam (GB) mode enhances resolution at low voltages by decelerating electrons just before they reach the specimen, allowing for detailed surface observation at energies as low as a few hundred eV. (Menon et al., 2019). This capability makes the FESEM ideal for imaging delicate, non-conductive materials like ceramics and semiconductors. Equipped with secondary and backscattered electron detectors, it provides comprehensive morphological and compositional analysis. (Fomina et al., 2006; Ye et al., 2023).

For the examination of the bio-cement generated by fungi isolates, field emission scanning electron microscopy (FESEM) is employed, as described by (Fang et al., 2018) wavelength range of 400-4000 cm^{-1} is utilized to delve into the structural intricacies, chemical properties, and bonding interactions of fungi hyphae with calcium ions.

5.9.4 Thermogravimetric Analysis (TGA)

To measure the change in mass of a material as a function of temperature or time under a controlled atmosphere. By heating a sample and recording its weight loss, TGA provides valuable insights into the thermal stability, composition, and decomposition

processes of the material. This method is widely used in materials science and chemistry to analyze polymers, composites, ceramics, and metals, helping researchers and manufacturers understand properties like moisture content, volatile components, and thermal degradation. Perkin Elmer's equipment is used for the same (Yu et al., 2022; Ditta et al., 2024).

5.10 Crack filling

For self-healing bio-cement cubes using fungi, cubes were prepared by inoculating a fungi culture into a nutrient medium that promotes spore germination and subsequent fungi growth. Specific fungi strain capable of calcium carbonate precipitation were selected for its efficiency in promoting self-healing through microbial-induced calcite precipitation (MICP). The fungi spores were first grown in a liquid medium containing urea and calcium chloride to produce calcium carbonate precipitates. Once the fungi culture matured, the spores and metabolic byproducts were incorporated into a cementitious mixture, which was then poured into cube moulds and allowed to set. After initial curing, cracks were made, allowing microfractures to develop. Over a 28-day incubation period, the fungi within the microcracks produced calcium carbonate, effectively sealing the cracks and restoring the structural integrity of the cubes. This methodology highlights the self-healing potential of bio-cement, offering an eco-friendly solution to traditional maintenance methods in construction materials (Jongvivatsaku et al., 2019; Kulkami et al., 2020)

5.11 Genotypic and Phylogenetic analysis

To identify the selected potential fungus 18s rRNA technique was performed in which DNA was isolated from the freshly grown plated fungi culture, and its quality was confirmed by a single high-molecular-weight band on a 1.0% agarose gel. The ITS gene fragment was amplified via PCR, yielding a distinct amplicon band on agarose gel, which was subsequently purified using column purification. Sequencing of the purified amplicon was performed using ITS1 and ITS4 primers with the BDT v3.1 Cycle Sequencing Kit on an ABI 3500xl Genetic Analyzer. The resulting ITS sequence was subjected to BLAST analysis against the NCBI GenBank database, and the top ten

sequences with the highest identity scores were aligned using multiple sequence alignment software.

A full methodology of research is given in Figure 5.4.

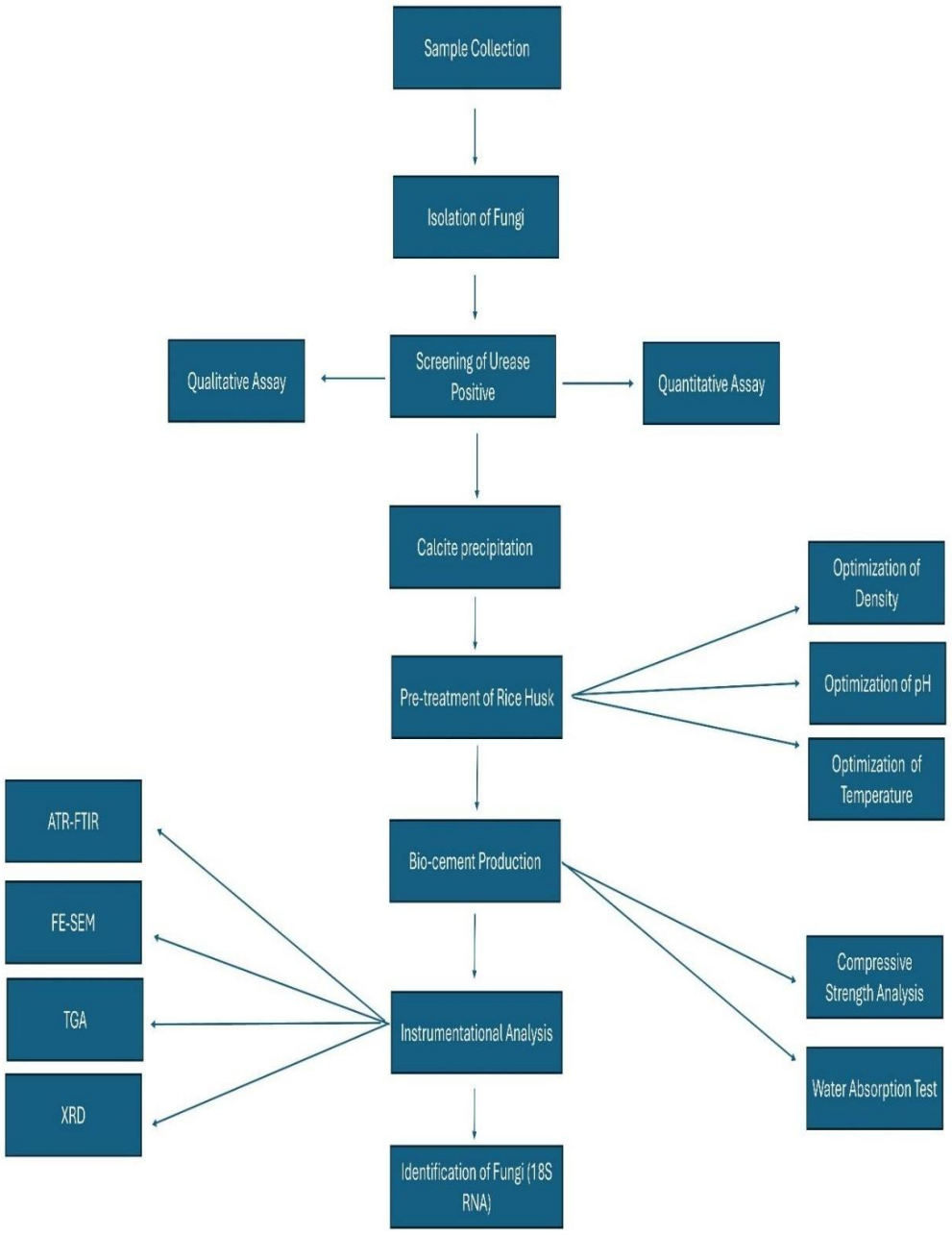


Figure 5.4: Methodology showing production of Bio-cement.

CHAPTER 6

RESULT AND DISCUSSION

6.1 Sample collection and isolation

A total of 46 fungi colonies were successfully isolated from soil samples collected across various alkaline sites (Chandini et al., 2017). These fungi isolates demonstrated diverse morphological characteristics, which were assessed to understand their potential functional roles in calcite precipitation. Colony colors ranged broadly, including shades from pure white, cream, and light yellow to deeper hues such as greenish-gray and brown, suggesting the presence of a wide variety of fungi genera. Texture variations were notable as well; some colonies exhibited a smooth, velvety surface, while others had a cottony or powdery appearance, and a few displayed a distinctly granular or irregular texture. These textures are often associated with specific types of fungi mycelium and sporulation structures, indicating morphological adaptations to their native environments (Alsohaili et al., 2018).

In addition to surface characteristics, growth rates also varied among the isolates. Some colonies expanded rapidly within the 5-7 day incubation period, covering a significant area on the agar plate, while others exhibited slower growth patterns, forming dense, compact colonies. These differences in growth rates and colony characteristics were critical in determining which isolates might be more suitable for bio-cement applications, as faster-growing, robust strains with dense structures are often preferred for practical applications in bio-cementation.

Microscopic examination further revealed variations in spore formation, hyphal structure, and cell wall thickness among the isolates. These structural features could influence each isolate's ability to precipitate calcium carbonate, as cell surface area and enzymatic activity are important factors in promoting calcite formation. Based on these preliminary observations, the isolates exhibited a promising range of phenotypic traits, indicating a rich diversity with potential applications in bioremediation, soil stabilization, and environmentally-friendly cementation processes.

The storage of fungi isolates was crucial to ensure the viability and integrity of the strains for future applications and studies. Short-term storage was achieved by transferring pure colonies onto PDA slants and maintaining them at 4°C, a temperature

that slows fungi metabolism without compromising cell viability. This approach provided a readily accessible means of preserving the isolates for immediate use and further analysis. PDA slants, which support sustained fungi viability were also used to preserve fungi for longer period of time at 4°C (Singh et al., 2018).

For long-term preservation, glycerol stocks of each isolate were prepared and stored at -80°C. Glycerol acts as a cryoprotectant, preventing ice crystal formation that can damage fungi cell walls during freezing (Rohadi et al., 2020; Webb et al 2018).

6.2. Urease assay

6.2.1 Qualitative Urease Test

In the qualitative urease test, the ability of fungi isolates to produce the enzyme urease was assessed using urea broth as an indicator medium (Figure 6.1). Urease is a key enzyme in microbial calcite precipitation processes, catalyzing the breakdown of urea into ammonia (NH_3) and carbon dioxide (CO_2) (Sigurdarson et al., 2020). This enzymatic reaction is critical because it results in the production of ammonia, which raises the pH of the surrounding environment, creating alkaline conditions. The urea broth medium includes phenol red, a pH-sensitive indicator that exhibits a clear color change in response to shifts in pH: it appears yellow under acidic conditions and transitions to pink as the medium becomes alkaline, specifically at a pH above 8.2 (Sigurdarson et al., 2020). When fungi capable of urease production hydrolyze urea, the resultant ammonia production causes the pH to increase, leading to the visible color shift from yellow to pink in the medium. This color change serves as a straightforward visual confirmation of urease activity.

In this study, all fungi isolates were subjected to the urease test to determine their capacity for enzyme production, with 46 isolates identified as urease-positive. This result underscores the high prevalence of urease-producing fungi within the collected samples, marking them as potential candidates for calcite precipitation. The presence of urease in these isolates suggests that they could effectively contribute to calcium carbonate formation through urea hydrolysis, a fundamental reaction in bio-cementation and bioremediation applications (Ambarsari et al., 2018). Following the

qualitative assessment, quantification of urease activity was pursued to gain insights into the relative efficiency of urease production among the isolates.

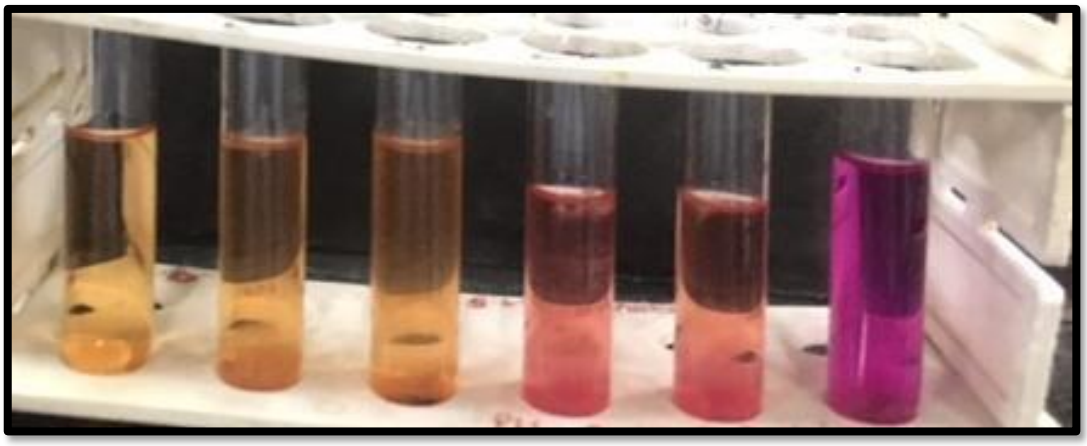


Figure 6.1: The colour change representing the production of urease enzyme.

6.2.2 Quantitative Urease Assay

For measurement of the urease enzyme standard graph of ammonium sulphate using the known value of ammonium sulphate is prepared to calcite the unknown amount for urease enzyme production (Figure 6.2, Figure 6.3).

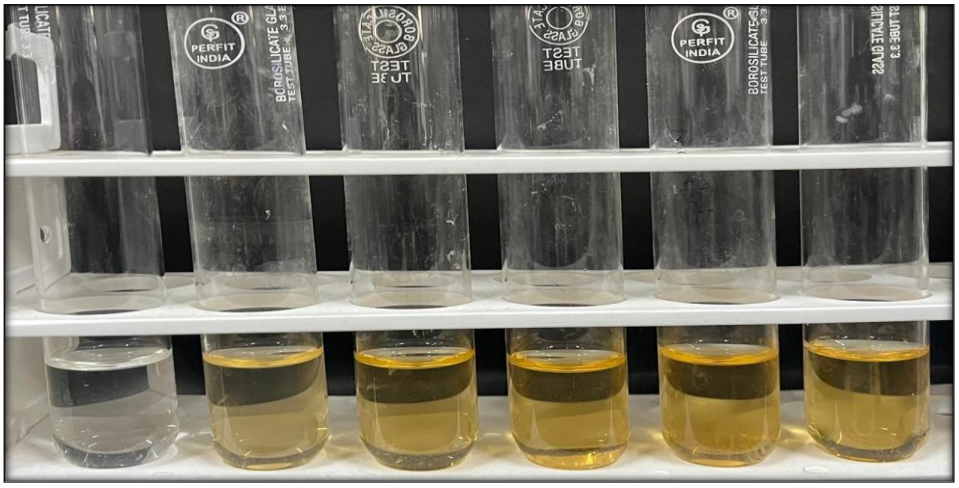


Figure 6.2: Preparation of standard solutions with varying concentrations (0-5 ppm) of ammonium sulphate dissolved in distilled water for standard graph.

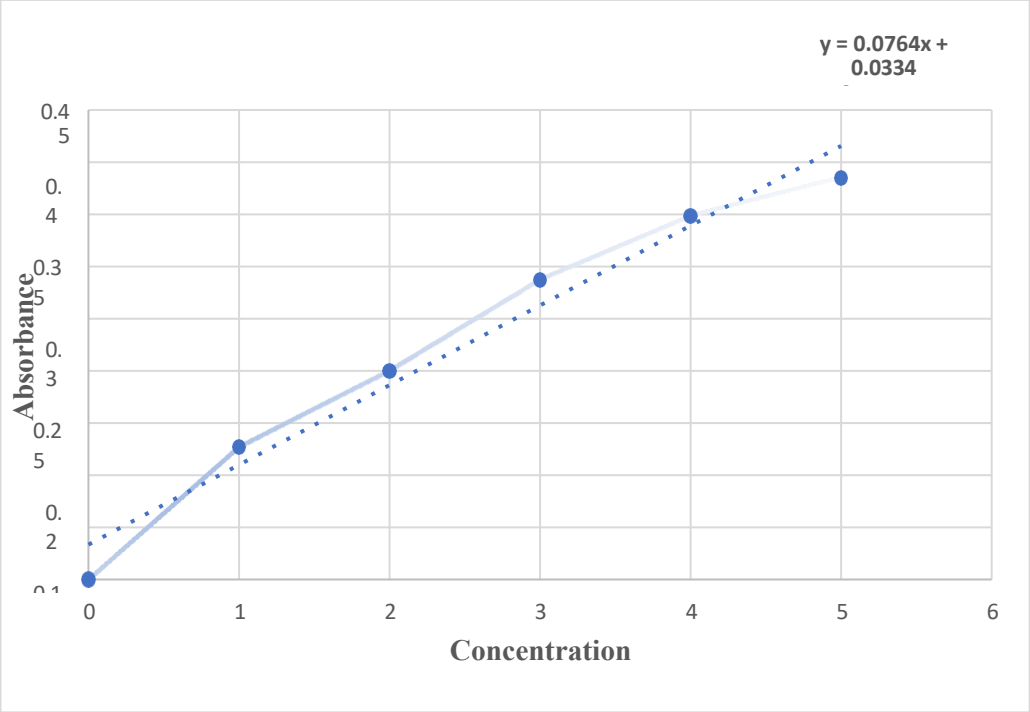


Figure 6.3 : Standard graph of ammonium sulphate absorbance recorded at 620 nm.

The quantitative urease assay results reveal a range of urease activities across the fungi isolates (table 6.1, figure 6.4), indicating different capacities for ammonia production and, by extension, urea hydrolysis. The concentration values range from lower activity at around 2.20 µg/ml to significantly higher activity at 10.06 µg/ml. This variation is indicative of the metabolic diversity within the isolates and highlights certain fungi as potentially more efficient for bio-cement applications due to their ability to elevate the surrounding pH and promote calcium carbonate precipitation more effectively.

Isolates with higher average concentrations, such as S4 (1), S4 (9), S1 (18), S6 (9), and S1 (3), demonstrated notably strong urease production, marking them as ideal candidates for applications where rapid or substantial calcite precipitation is needed. The precision of these results was enhanced by calculating the standard deviation, as

seen in the ± values, which further confirmed the consistency in activity among replicates.

Table 6.1: Urease enzyme produced in ug/ml by different urease-positive fungi.

Sr. No.	Fungus	Concentration 1 (mm/ml)	Concentration 2(mm/ml)	Concentration Avg. (mm/ml)
1	S5(9)	3.054973822	1.353403141	2.204188±0.85
2	S5(6)	3.054973822	2.662303665	2.858639±0.19
3	S6(1)	1.746073298	3.971204188	2.858639±1.11
4	S6(14)	3.316753927	2.531413613	2.924084±0.39
5	S2(7)	2.92408377	3.709424084	3.316754±0.39
6	S2(4)	3.185863874	3.840314136	3.513089±0.32
7	*Penicillium chrysogenum*	4.494764398	4.363874346	4.429319±0.06
8	S5(3)	5.672774869	3.709424084	4.691099±0.98
9	S5(10)	4 .232984293	5.541884817	4.887435±0.65
10	S4(3)	4.363874346	5.280104712	4.82199±0.45
11	S6(7)	4.363874346	5.280104712	4.82199±0.45
12	S5(1)	4.887434555	5.541884817	5.21466±0.32
13	S6(16)	7.243455497	6.065445026	6.65445±0.58
14	S6(8)	7.243455	7.767016	7.505236±0.26
15	S1(28)	9.59947644	6.065445026	7.832461±1.76
16	S4(9)	8.945026178	6.719895288	7.832461±1.11

17	F	8.945026178	7.636125654	8.290576±0.65
18	S6(9)	10.90837696	8.945026178	9.926702±0.98
19	s1(18)	8.290575916	8.552356021	8.421466±0.13
20	S1(3)	7.934555	9.824461	8.879581±0.94
21	S4(1)	9.59947644	10.51570681	10.05759±0.45

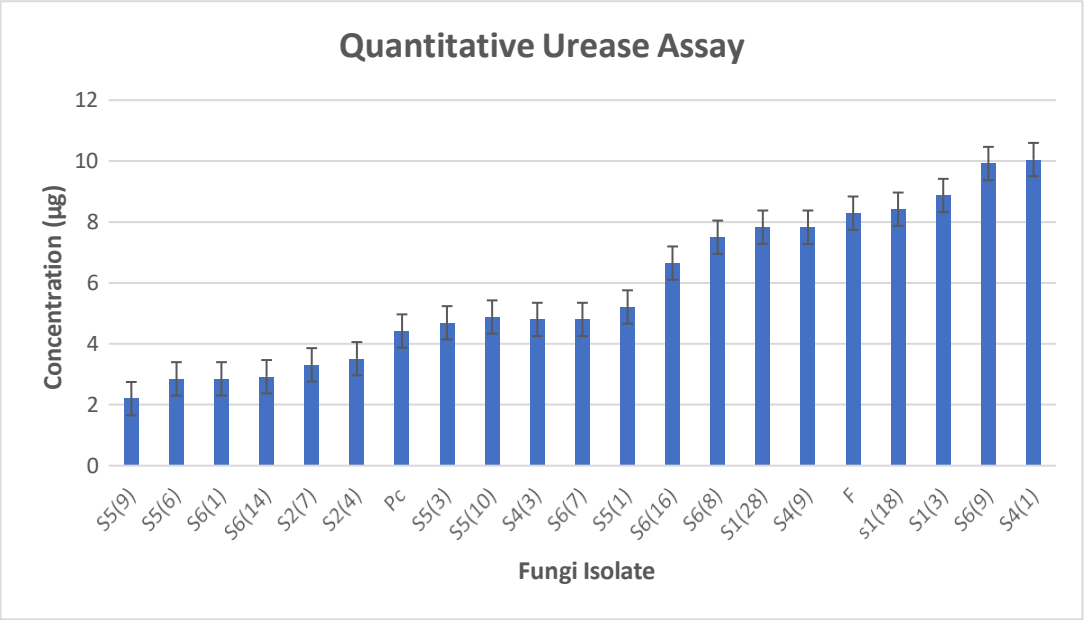


Figure 6.4: The Urease enzyme production in different fungi isolates.

Table Analyzed	Urease assay				
Data sets analyzed	A-U				
ANOVA summary					
F	10.76				
P value	<0.0001				
P value summary	****				
Significant diff. among means (P < 0.05)?	Yes				
R squared	0.9111				
Brown-Forsythe test					
F (DFn	DFd)	1.295e+030 (20	21)		
P value	<0.0001				
P value summary	****				
Are SDs significantly different (P < 0.05)?	Yes				
Bartlett's test					
Bartlett's statistic (corrected)					
P value					
P value summary					
Are SDs significantly different (P < 0.05)?					
ANOVA table	SS	DF	MS	F (DFn	DFd)
Treatment (between columns)	246.4	20	12.32	F (20	21) = 10.76
Residual (within columns)	24.04	21	1.145		
Total	270.5	41			
Data summary					
Number of treatments (columns)	21				
Number of values (total)	42				

Figure 6.5: The figure represents the statistical analysis of urease assay results using one-way ANOVA to determine significant differences among experimental groups.

6.3 Calcium Precipitation Assay

Calcium precipitation by urease-positive fungi is a bio-mediated process that holds significant potential for applications in sustainable construction materials. This process relies on the ability of specific fungi strains to hydrolyze urea, a reaction catalyzed by the enzyme urease, which the fungi produce (Dhami et al., 2017). During hydrolysis, urea breaks down into ammonium and carbonate ions, creating an alkaline microenvironment conducive to calcium carbonate ($CaCO_3$) precipitation when calcium ions are present. This biogenic calcium carbonate forms a crystalline structure that effectively binds substrate particles, such as sand or soil, contributing to the stability and strength of bio-cement (Mori et al., 2021).

The efficiency of calcium precipitation depends on factors like the concentration of urea and calcium ions, fungi growth rate, and environmental conditions such as pH and temperature. The urease-positive fungi used in this study, selected for their high urease activity, were instrumental in creating sufficient carbonate ions necessary for consistent calcium carbonate formation. The data for each isolate, presented as burette readings and their respective averages, illustrates the diversity in calcite-precipitating abilities among the fungi strains (Table 6.2, Figure 6.6). In this assay, lower burette readings correspond to higher calcium precipitation, as these values indicate a greater consumption of calcium ions due to effective calcite formation. Conversely, higher burette readings represent reduced calcite precipitation, suggesting that the fungi isolates were less effective at utilizing calcium ions for carbonate precipitation.

Isolates such as S1(3), S4(9), S1(18), S2(7), S1(28), S6(9) showed the lowest average burette values, with averages of 2.3379±0.57, 2.7044±0.27, 3.3398±0.57, 4.0746±0.11, 7.7056±0.57 µg/ml, respectively shown in Figure 6.6. These low readings signify that these isolates have strong calcite precipitation capabilities, effectively utilizing calcium ions in the solution to form stable calcium carbonate. Whereas, *Penicillium chrysogenum* showed 4.4628±0.33 µg/ml. Such isolates are prime candidates for applications in bio-cementation, as they demonstrate consistent calcium precipitation, which is crucial for binding particles and creating a structurally sound matrix in bioconstruction processes.

1 mL EDTA×Molarity EDTA=mmoles EDTA=mmoles Ca_2^+×40.078 g/mole=mmoles Ca_2^+,1mL EDTA×Molarity EDTA=mmoles EDTA=mmoles Ca_2^+×40.078g/mole=mmoles Ca_2^+,

aliquot 2(Ca_2^+ mg, aliquot)×(250.00 mL50.00 mL)=Ca_2^+ mg, unknown.aliquot 2(Ca_2^+ mg, aliquot)×(50.00mL250.00mL)=Ca_2^+mg, unknown.

Table 6.2: Calcium precipitation data of fungi isolates

Sr. No.	Fungi	Burette 1 (µg/ml)	Burette 2 (µg/ml)	Burette 3 (µg/ml)	Average (µg/ml)
1.	S4(3)	34.0663	40.078	38.0741	37.4061±3.06

2.	S5 (10)	32.0624	30.0585	29.05655	30.3925±1.53
3.	S5 (1)	22.0429	38.0741	30.0585	30.0585±8.02
4.	S5 (9)	14.0273	14.0273	16.0312	14.6953±1.15
5.	*Penicillium chrysogenum*	4.07674	4.6553	4.6565	13.3593±0.33
6.	S6 (16)	19.03705	16.0312	17.03315	13.3593±1.53
7.	S6 (14)	12.0234	14.0273	14.0273	13.3593±1.16
8.	S6 (8)	14.0273	10.0195	13.02535	12.3574±2.08
9.	S6 (7)	14.0273	8.0156	12.0234	11.3554±3.06
10.	S6 (1)	10.0195	10.0195	11.02145	10.3535±0.58
11.	S5 (3)	8.0156	10.0195	11.02145	9.6855±1.53
12.	S2(4)	4.0078	8.0156	10.0195	9.3515±1.15
13.	S2(4)	10.0195	8.0156	10.0195	9.3515±1.15
14.	S5 (6)	9.01755	8.0156	10.0195	9.0176±1.00
15.	S6 (9)	8.0156	8.0555	7.0457	7.7056±0.57
16.	S1(28)	3.00585	5.00975	5.00975	4.3418±1.1
17.	S2(7)	4.0078	4.20819	4.0078	4.0746±0.11
18.	S1(18)	3.00585	3.00585	4.0078	3.3398±0.57
19.	S4(9)	2.5039	2.6039	3.00546	2.7044±0.27
20.	S1(3)	2.0039	2.0039	3.00585	2.3379±0.57

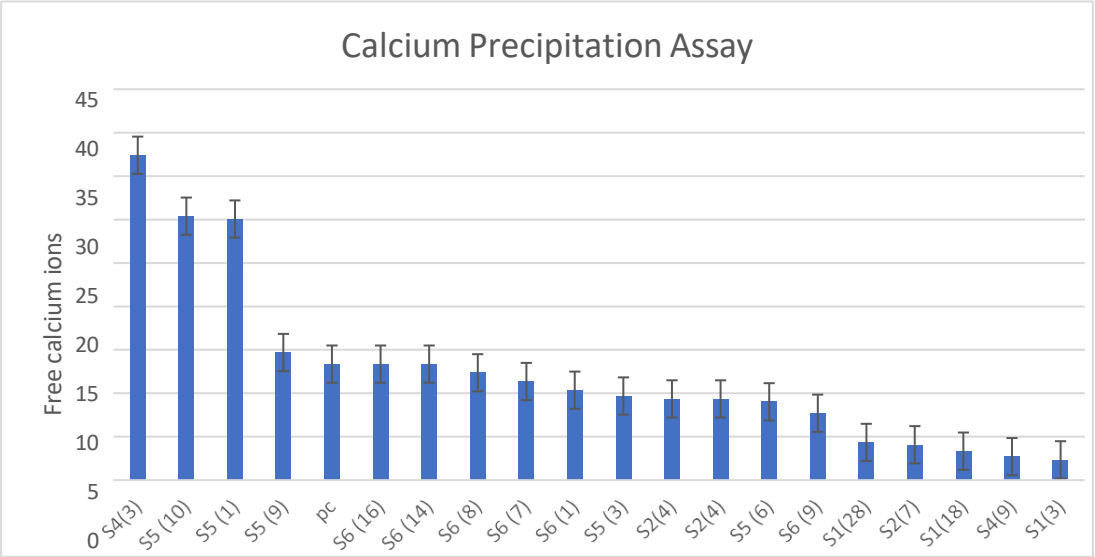


Figure 6.6: Calcium precipitation (µg/mL) by selected urease-positive fungi. Data represent mean ± SD of triplicates showing variation in biomineralization potential among isolates.

Table Analyzed	Calcium precipitation					
Data sets analyzed	A-S					
ANOVA summary						
F	52.76					
P value	<0.0001					
P value summary	****					
Significant diff. among means (P < 0.05)?	Yes					
R squared	0.9615					
Brown-Forsythe test						
F (DFn	DFd)	1.870 (18	38)			
P value	0.0518					
P value summary	ns					
Are SDs significantly different (P < 0.05)?	No					
Bartlett's test						
Bartlett's statistic (corrected)						
P value						
P value summary						
Are SDs significantly different (P < 0.05)?						
ANOVA table	SS	DF	MS	F (DFn	DFd)	P value
Treatment (between columns)	5512	18	306.2	F (18	38) = 52.76	P<0.0001
Residual (within columns)	220.6	38	5.804			
Total	5733	56				
Data summary						
Number of treatments (columns)	19					
Number of values (total)	57					

Figure 6.7: The figure represents the statistical analysis of calcium precipitation assay results using one-way ANOVA to determine significant differences among experimental groups.

6.4 Rice straw processing

6.4.1 Collection of Rice straw

The rice straw preparation process was designed to ensure purity, uniformity, and quality control, which are essential for reproducible results in further chemical treatments. Washing the straw minimized the potential for contamination from external impurities, such as soil particles or microbial residues, which could interfere with subsequent reactions. Drying the straw at 50°C was essential to remove residual moisture, as water content could impact the consistency and effectiveness of future chemical treatments. Grinding the straw into a fine powder and sieving it through a 1 mm mesh allowed for uniform particle size, enhancing the surface area and ensuring that the chemical treatments would be evenly distributed throughout the material. The airtight storage further safeguarded the processed powder from moisture absorption and contamination, maintaining the integrity of the rice straw for consistent results in upcoming experiments.

6.4.2 Chemical Pretreatment

After the delignification process, the treated rice straw, now with reduced lignin content and increased accessibility to cellulose and hemicellulose, was subjected to further biochemical and instrumental analyses to confirm successful processing.

6.4.3 Filtrate Analysis

6.4.3.1 Molisch's Test

The presence of any carbohydrates released after the pretreatment of rice straw has been checked in the pretreated rice straw extracts shown in Figure 6.8. The purple ring formation confirmed that carbohydrates are present in various pretreated rice straw extracts using Molisch's test.

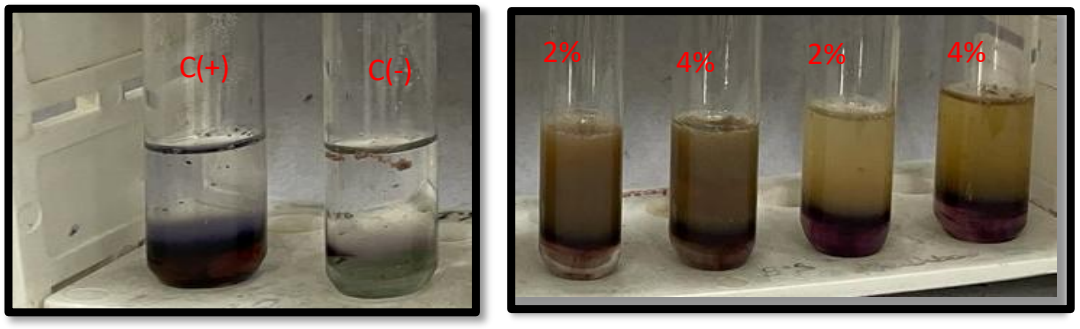


Figure 6.8: Molisch's test determination of the presence of carbohydrates after the pretreatment of rice straw.

4.3.2 DNS test

This test helps in calculating total reducing sugars after the pretreatment of RS using differing NaOH and H_2SO_4 in the triplet. A standard glucose curve is prepared to calculate sugar released after treatment with sulfuric acid for quantitative analysis of sugar present in the extract shown in Table 6.3.

Table 6.3: Concentration of X (µg/mL) derived from standard glucose graph.

Rice straw treatments	Treatment process	Absorbance (Avg.)	Sugar concentration (µg/mL)
R-1	2% NaOH	0.294	0.9±0.0008
R-2	4% NaOH	0.51633	1.7±0.0012
R-3	2% H_2SO_4	1.321	4.4±0.004
R-4	4% H_2SO_4	1.2	4.1±0.0008

All the treatment process results are calculated for sugar extracted using the formula from the standard glucose curve:

X = (Y+0.0004)/0.0003

Here X represents an independent variable (µg/mL) (unknown concentration of sugar) and Y is the dependent variable (known concentration (µg/mL) of sugar). The formula or equation is derived from the standard glucose curve. The treatment processes using 2% NaOH yielded 0.189 µg/mL sugar of the extract, 4% NaOH yielded 0.359 µg/mL sugar of the extract, 2% H_2SO_4 yielded 5.99 µg/mL sugar of the extract, and lastly, 4% H_2SO_4 yield 3.13 µg/mL sugar of the extract. These results suggest that sulfuric acid treatments, particularly at 2%, were more effective in releasing sugars, highlighting its role as a strong agent in the delignification and hydrolysis of rice straw.

6.4.3.3 Fourier transform Infrared spectroscopy (FTIR)

There are noticeable alterations in the proportions of rice straw components such as hemicellulose, cellulose, and lignin (Figure 6.9). It is determined that the O-H stretching vibration is present in the lignin component since it exhibited an intense band with a broad frequency range between 3100 and 3500 cm^{-1}. In its natural condition, Rice straw had a peak in the infrared spectrum at 3409.26 cm^{-1}, indicating the existence of phenolic and alcoholic hydroxyl groups. When rice straws are treated with sodium hydroxide at 3%, 5%, and 7%, the resulting wavenumber shifts are 3410.31 cm^{-1}, 3429.48 cm^{-1}, and 3425.09 cm^{-1}, respectively. It is determined that these shifts are caused by the demethylation of the O-CH3 bond present in the methoxy group, which resulted in the substitution of CH3 and the production of a new O-H group. According to Tsegaye et al. (2019), following the 3% and 7% treatments, the aromatic ring peaks identified at 2360.43 cm^{-1} and 2330.99 cm^{-1} disappeared after exposure to those treatments' concentrations. Note that the crystalline structure of the biomass remained unaffected by the dilute acid pretreatment at 121°C, explaining the similarity in FTIR patterns between the native and dilute acid-pretreated rice straw. The processed biomass exhibited prominent peaks at 809, 901, 1160, 1249, 1320, 1373, 1439, 1512, 1640, 1726, 1879, 2130, 2346, and 2917 cm^{-1}, whereas the native

biomass showed peaks at 808, 1170, 1365, 1430, 1516, 1643, 1879, 2140, and 2924 cm-1 cm^{-1}. The authors attributed the absence of the 1725 cm^{-1} peak in acid-pretreated rice straw to the acid hydrolysis of hemicelluloses present in the natural straw. The surface structure of the biomass, as observed in the FTIR spectrum patterns, appeared to have undergone similar changes in both native and dilute acid-processed rice straw, according to Kshirsagar et al. (2015). Kumari and Singh (2022) observed that the first peak at 786 cm^{-1} in the FTIR spectra, attributed to the C-H group in lignin and its out-of-plane vibration, exhibited reduced transmittance, particularly in rice straw treated with alkaline wastewater compared to distilled water-treated straw. The reduction in transmittance indicated a decrease in lignin removal and highlighted the effectiveness of alkaline wastewater treatment for rice straw pretreatment. The elimination of cellulose and hemicellulose, along with their connection to lignin and each other, decreased transmittance for C-H deformation and C-O stretching in alkaline wastewater-treated rice straw. A comparison between native rice straw and distilled water-treated straw showed increased transmission in the latter, indicating reduced lignin removal.

Furthermore, the authors noted a decrease in the strength of the lignin-related absorption band at 1546 cm^{-1} in the spectra of alkali-treated rice straw. After bleaching, the absorption peak at 808 cm^{-1}, attributed to C-H aromatic hydrogen, is absent in rice straw and alkali-treated rice straw. The peaks at 1156 and 1160 cm^{-1} are attributed to the pyranose ring skeletal C-O-C bonds of cellulose, while the significant peak of the hemicellulose and cellulose structures are linked to the significant peak at 1040 cm-1 cm^{-1} (Musa et al., 2017).

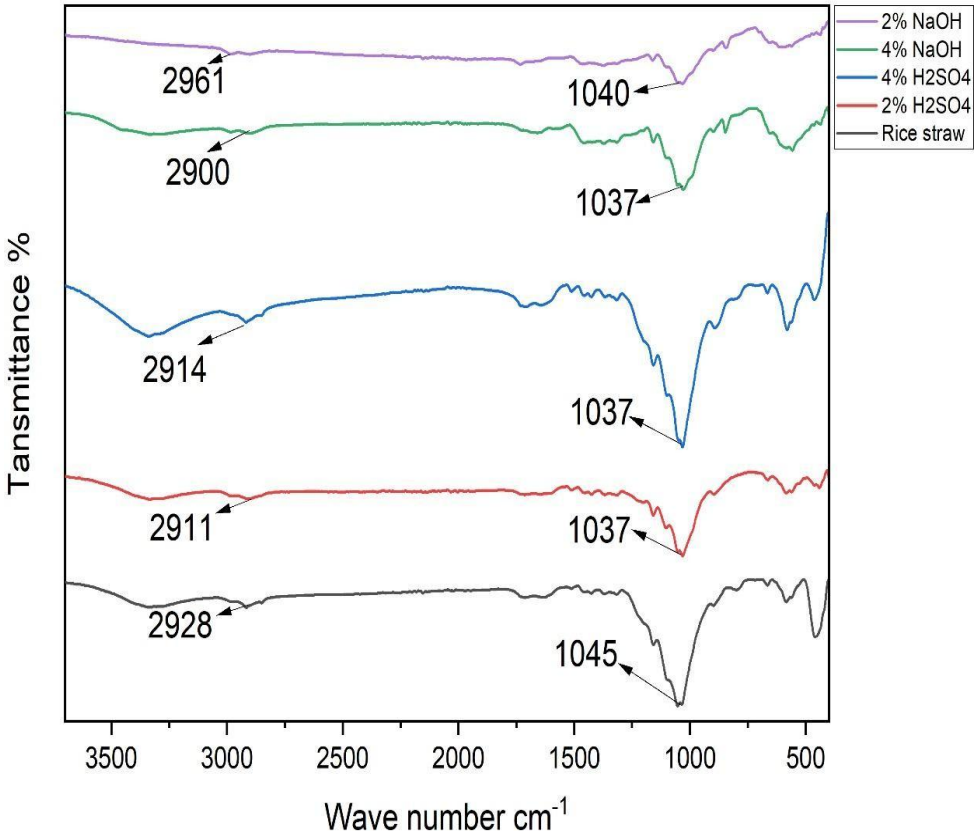


Figure 6.9: FTIR of rice straw (raw and chemically treated).

6.4.3.4 XRD of rice straw (raw and chemically treated)

The XRD spectra of raw and different pretreated rice straws (Figure 6.10). The amount of crystalline matter in biomass can be determined by calculating the crystallinity index (CI) and the average crystallite size. The crystallite size is calculated for all the pretreatments. The crystallite size for raw RS is found to be 0.72.

Similarly, when treated with a 2% H_2SO_4 solution, the crystallite size increased to 2.72 , 4% H_2SO_4 treatment, the crystallite size increased to 1.75. In contrast, applying a 2% NaOH treatment resulted in a crystallite size of 1.38, whereas a 4% NaOH treatment yielded a slightly larger crystallite size of 1.4. Depending upon the size of crystallinity of different treated RS, the maximum size of 2% H_2SO_4 treatment is noted compared to other treatments and raw RS. The observed spectra of raw rice straw are three distinct peaks in the XRD spectrum corresponding to crystalline cellulose. The work by the

authors calculated CI for untreated rice straw as 42%, but it is shown to rise to 56% after acid treatment. This finding is consistent, acid treatment eliminates accessible amorphous cellulosic and amorphous hemicellulose areas, increasing the substrate's CI. As evidence that alkali treatment reduces cellulose's CI by eliminating resistant lignin and subsequently opening the substrate's structure, further alkali treatment resulted in a CI decrease of 40% (Kaur and Kuhad, 2019). The authors analysed the XRD of untreated straw, which had a CI value of 66.27%; this value was reduced to 48.67% after being treated with acid and increased to 55.97% after being treated with alkali. The fragmentation of the crystalline structure of the cellulose component is determined to be the cause of the decrease in CI found in the samples that had been processed. The results demonstrate that the pretreatment has a considerable effect on the crystallinity of the cellulose. However, in this study, the authors concluded that the CI value of the alkali-treated straw reduced is higher than that of the acid-treated straw because of the lignin component's destruction and removal during the previously stated alkali pretreatment. The increased CI in alkali-treated biomass is linked to retaining the biomass' crystalline cellulose while the amorphous areas comprising the hemicellulose and lignin fractions are removed (Mechery et al., 2021, Boonsombuti et al., 2020, Chiranjeevi et al., 2018).

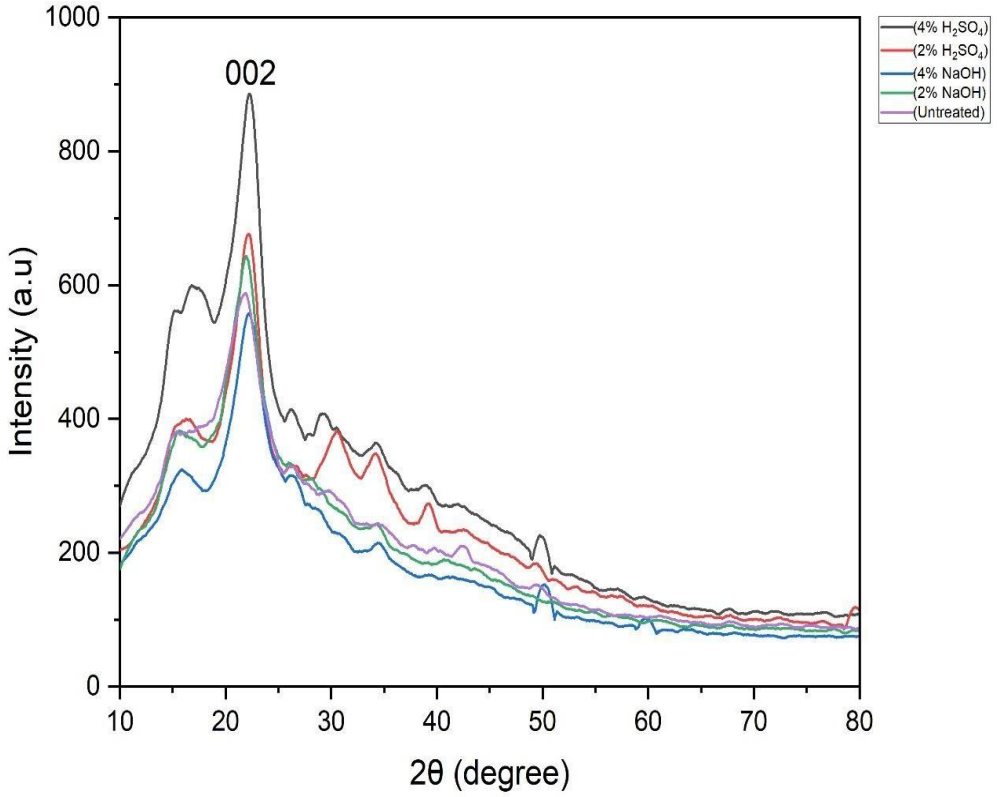


Figure 6.10: XRD diffractogram of rice straw (raw and chemically treated)

6.5 Optimization of 2% H_2SO_4 pretreated extract for fungi growth

Various parameters were optimized to achieve maximum fungi biomass yield using a one-factor-at-a-time (OFAT) optimization approach. Parameters including inoculum density, pH, and temperature were systematically adjusted to determine optimal conditions (Singh et al., 2017)

6.5.1 Density Optimization

Optimization of chemically treated rice straws after autoclaving and preparation of various 100%, 75%, 50%, and 25% dilutions are prepared in triplet, the fungi samples are inoculated for seven days, and after incubation, biomass is calculated using Whatman filter paper. The Whatman filter paper is dried for 24 hours, and the weight

of the 7-day-old incubated cultures is determined using the Whatman filter paper. After a 24-hour drying period, the weight of the Whatman filter paper is measured, and 7-day-old incubated cultures are filtered and dried to determine the dry weight of the fungi cultures. A 100% rice straw extract concentration showed maximum fungus growth as the maximum dry weight was measured, as depicted in Figure 6.11. Density optimization of the pretreated rice straw that showed maximum growth of S1(3), S4(9), and S6(9) fungi isolate at 100% concentration (table 6.4). So, based on the statistical p-values, fungi type ($p < 0.05$) showed no statistically significant effect on biomass production, whereas density ($p > 0.05$) had a highly significant effect, indicating that biomass yield is strongly influenced by density but not by the fungi strain.

Table 6.4: Density optimization of S1(3), S4(9), and S6(9).

Sr. No	**Fungi**	**Density**	**Biomass**			**Average**
1	**S1(3)**	25	0.12	0.14	0.11	0.12333±0.015
		50	0.19	0.21	0.2	0.196667±0.01
		75	0.32	0.33	0.3	0.31±0.015
		100	0.35	0.34	0.32	0.33667±0.015
2	**S4(9)**	25	0.24	0.25	0.24	0.24333±0.015
		50	0.24	0.2	0.21	0.216667±0.02
		75	0.25	0.25	0.27	0.256667±0.01
		100	0.48	0.5	0.45	0.476667±0.025
3	**S6(9)**	25	0.14	0.14	0.16	0.146667±0.011
		50	0.21	0.24	0.19	0.21333±0.025
		75	0.34	0.29	0.27	0.3±0.030
		100	0.55	0.45	0.5	0.5±0.05

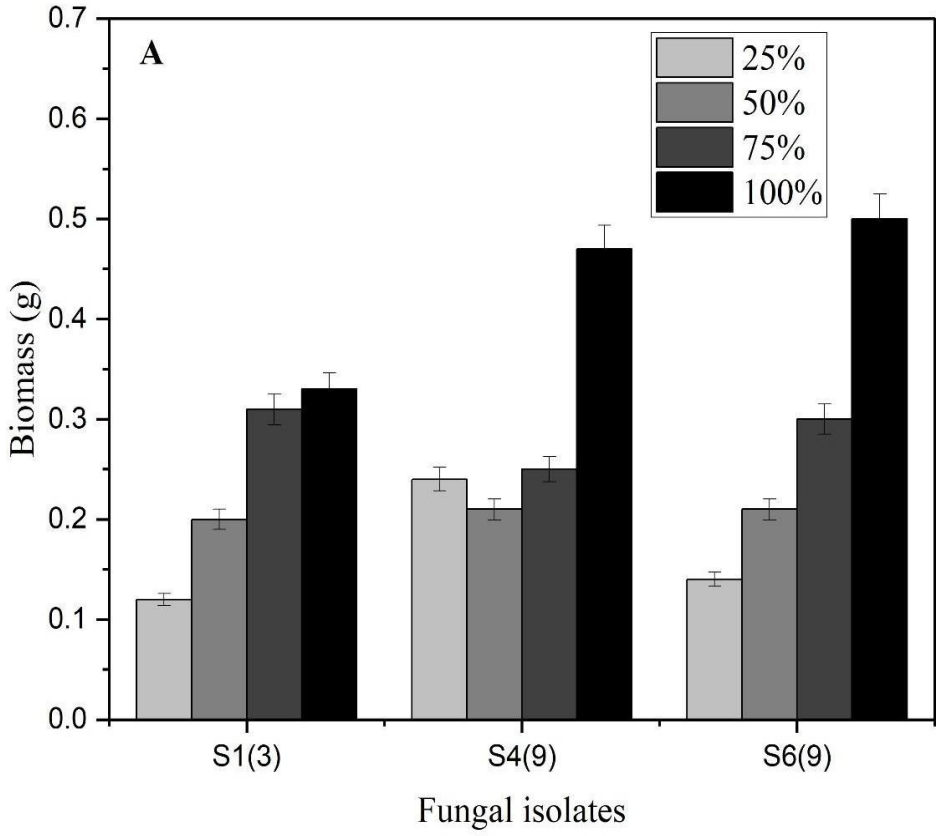


Figure 6.11: Graph A represents the optimization of 2% H_2SO_4 pretreated rice straw for maximum fungi growth at different densities.

6.5.2 pH Optimization

After autoclaving, the chemically treated rice straws are optimized. Different pH values 8, 9, 10, and 11 are adjusted in triplet, same protocol is followed as explained above. S1 (3) gave the most significant biomass weight at pH 9, S4 (9) at pH 8, and S6 (9) at pH 9 gave the maximum dry weight, as depicted in Table 6.5 and the graph for the same in figure 6.12. However, based on the p-values, fungi type ($p = 0.00018$) showed a statistically significant effect on biomass production across all pH levels, whereas pH ($p = 0.0587$) did not have a statistically significant effect at the 5% level, though it was close to significance, indicating a potential trend.

Table 6.5: pH optimization of S1 (3), S4 (9), and S6 (9).

Sr. No.	Fungi	pH	Biomass			Average
1.	**S1 (3)**	8	0.27	0.27	0.4	0.3133±0.07
		9	0.52	0.65	0.73	0.6333±0.10
		10	0.19	0.5	0.22	0.30333±0.17
		11	0.2	0.12	0.23	0.18333±0.05
2.	**S4 (9)**	8	0.24	0.18	0.21	0.21±0.03
		9	0.19	0.17	0.17	0.176667±0.01
		10	0.12	0.22	0.14	0.16±0.05
		11	0.06	0.08	0.08	0.07333±0.01
3.	**S6 (9)**	8	0.14	0.11	0.13	0.126667±0.015
		9	0.15	0.2	0.18	0.176667±0.025
		10	0.11	0.22	0.11	0.146667±0.063
		11	0.13	0.18	0.17	0.16±0.026

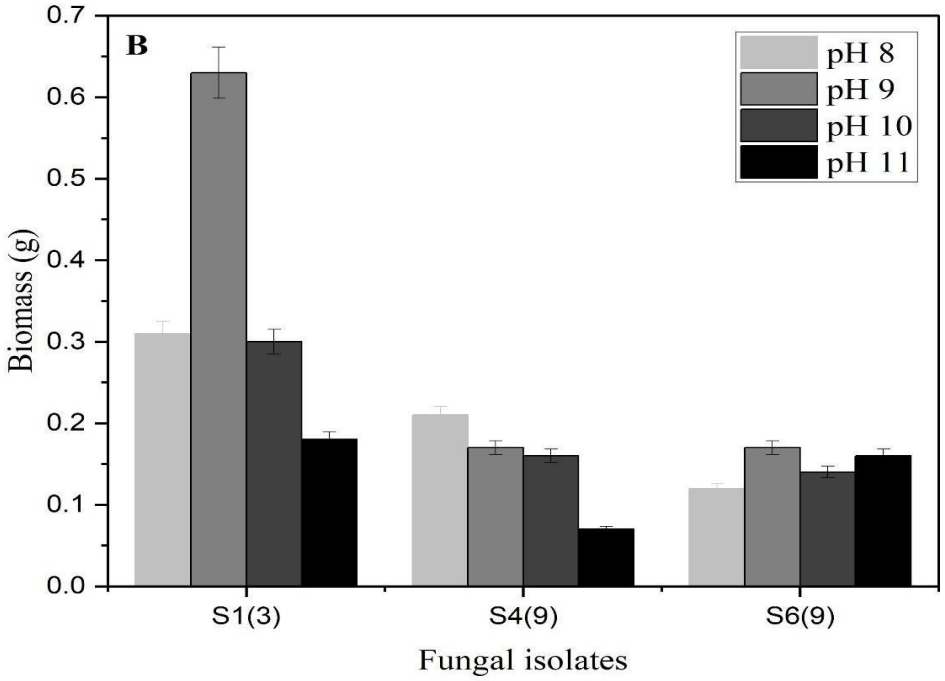


Figure 6.12: Graph B represents the optimization of 2% H_2SO_4 pretreated rice straw for maximum fungi growth at different pH.

6.5.3 Temperature Optimization

Table 6.6: Temperature optimization of S1(3), S4(9), and S6(9)

Sr. No.	Fungi	Temperature	Biomass			Average
1.	**S1 (3)**	20	0.09	0.13	0.11	0.11±0.02
		25	0.16	0.17	0.17	0.16±0.005
		30	0.36	0.36	0.36	0.36±0
2.	**S4 (9)**	20	0.18	0.16	0.14	0.16±0.005
		25	0.2	0.22	0.21	0.21±0.01
		30	0.22	0.23	0.24	0.25±0.02
3.	**S6 (9)**	20	0.13	0.14	0.15	0.14±0.01
		25	0.2	0.19	0.21	0.2±0.01
		30	0.23	0.25	0.27	0.25+0.02

After optimizing the density and pH of the extract, the next factor optimized is temperature. Optimized density, i.e., 100%, is prepared, and optimized pH is maintained for strains. To optimize the temperature, S1 (3) is adjusted to pH 8, S1(18) to pH 10, and S4 (1) to pH 9, and the rice extract is autoclaved at 121 ºC at 103 kPa for 15 min. Then, inoculation is performed, and samples are incubated at different temperatures of 20º C, 25º C, and 30º C for seven days in the triplet (table 6.6). After a 24-hour drying period, the weight of Whatman filter paper is measured to determine the dry weight of the fungi cultures, as depicted in Figure 6.13. The 2% H_2SO_4- pretreated rice straw extract at 100% concentration, pH 9–10, and 30°C yielded the highest fungi biomass. So, based on the p-values, fungi type ($p > 0.05$) had no statistically significant effect on biomass production, whereas temperature ($p < 0.05$) had a highly significant effect, indicating that biomass yield was strongly influenced by temperature but not by the fungi strain. These optimized conditions are ideal for promoting fungi growth and will facilitate further studies on utilizing rice straw as a sustainable substrate in biotechnological applications.

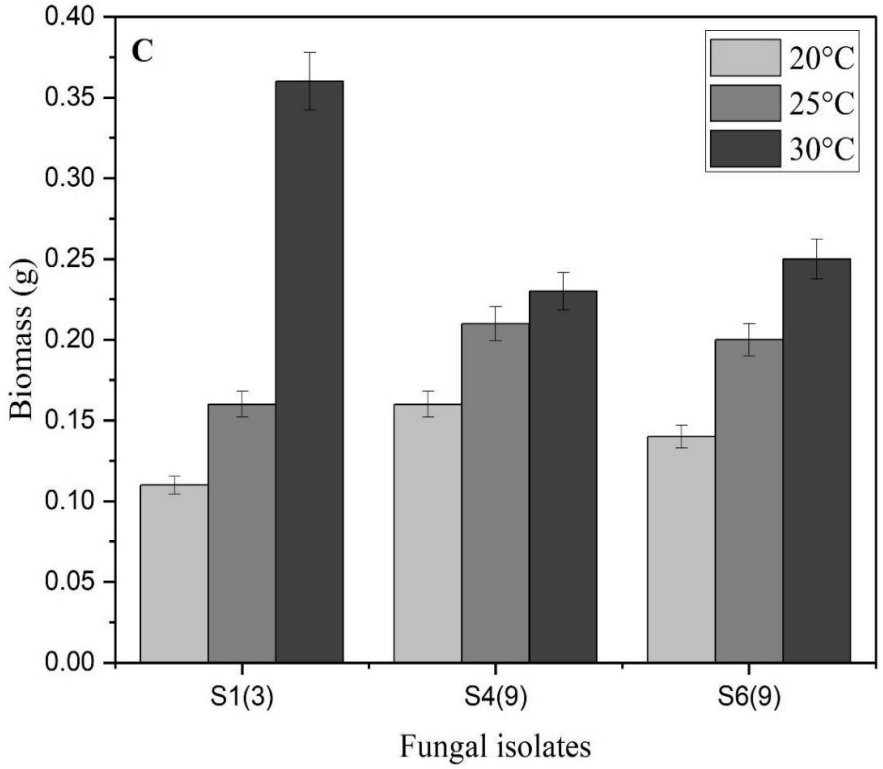


Figure 6.13: Graph C represents the optimization of 2% H_2SO_4 pretreated rice straw for maximum fungi growth at different temperatures.

6.6 Production of Bio-cement using fungi

In the laboratory production of bio-cement bricks, an aluminum foil container was used as a mold for shaping and containing the bio-cement mixture shown in Figure 6.14. The process began by preparing a homogenous mixture of fungi extract, urea, calcium chloride, following a standard protocol. After thorough mixing, the bio-cement slurry was poured into the aluminum foil container and left to solidify. This container provided an inexpensive, easily mouldable, and non-reactive environment for shaping the bio-cement while allowing the slow evaporation of moisture, essential for proper hardening. Over a 10-day incubation period at controlled temperature 27ºC, fungi-induced mineralization occurred, leading to the formation of a solid bio-cement structure. After drying in a hot air oven, the resultant bio-cement brick displayed promising structural

integrity, confirming the suitability of aluminum foil containers as practical moulds for laboratory-scale bio-cement production.

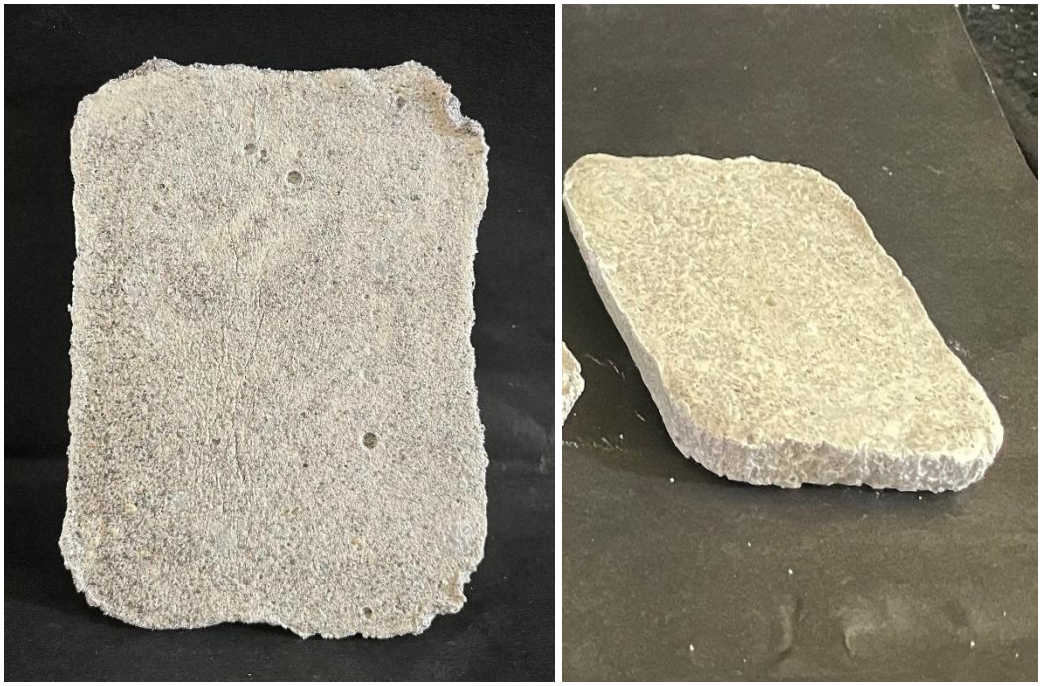


Figure 6.14: Prepared laboratory scale bio-cement bricks using fungi

6.7 Scale up production

In the scale-up production of bio-cement bricks, large moulds measuring 7 cm × 7 cm were successfully utilized, resulting in structurally solid and durable bio-cement bricks (Figure 6.15). The mixture consisted of sand, calcium chloride, urea, 100% optimized rice straw extract, and freshly cultivated fungi biomass. These components were thoroughly mixed, ensuring a consistent medium for fungi growth and even mineralization within the moulds. This drying step removed the moisture, leaving behind a durable bio-cement brick. This pilot-scale procedure demonstrated the role of each component, from calcium and urea as core reactants to the fungi as bio-catalysts, in creating a scalable, environmentally-friendly building material.

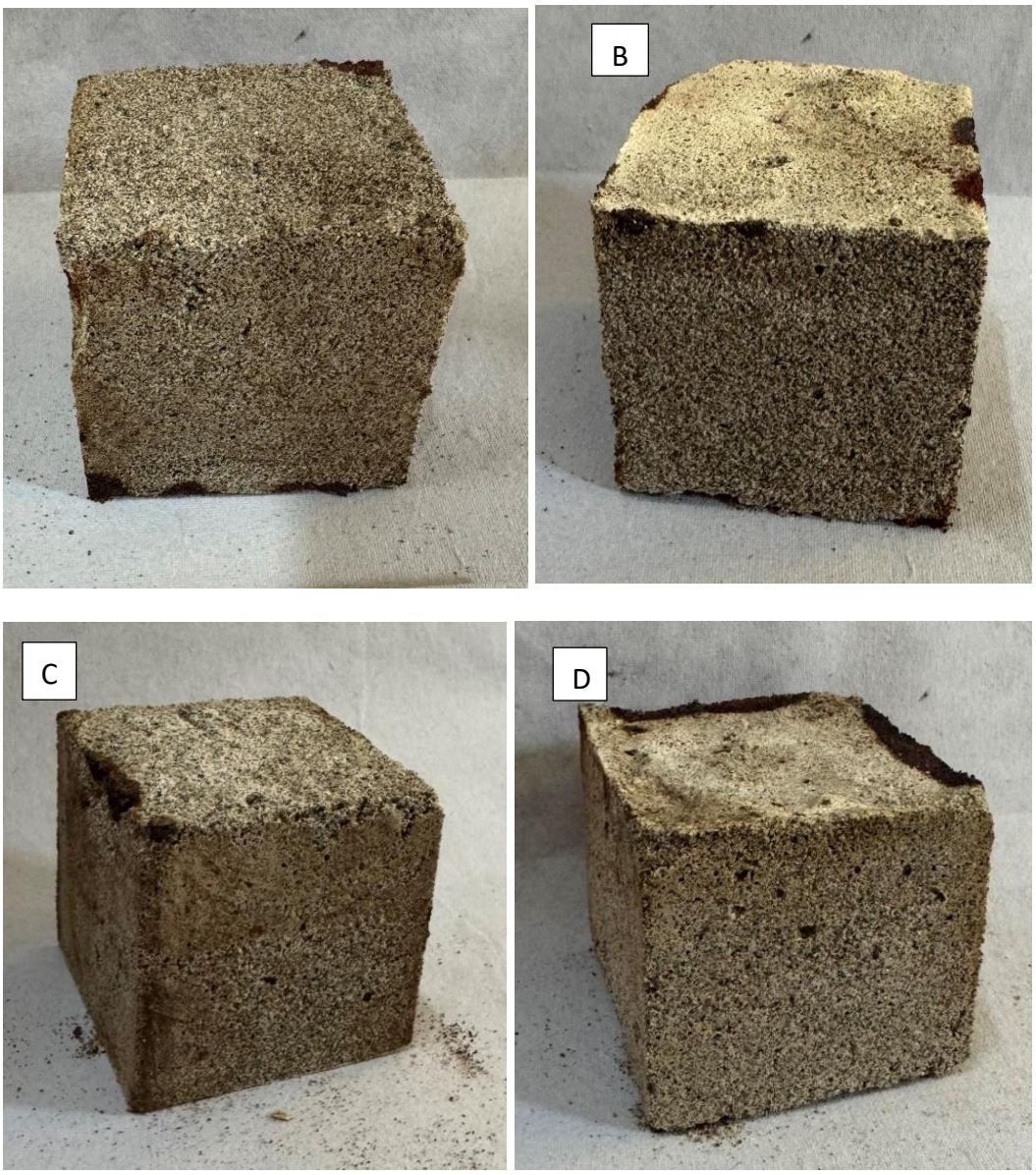


Figure 6.15: Bio-brick prepared using control *Penicillium chrysogenum* and other selected urease-positive fungi where A, B, C, and D represent *Penicillium chrysogenum*, S1 (3), S4 (9), and S6 (9) respectively

6.8 Bio-cement analysis

6.8.1 Strength Analysis

The strength analysis of bio-bricks produced through fungi-induced calcium carbonate precipitation was assessed using a compression testing machine. This test provided crucial insights into the compressive strength and durability of the bio-bricks compared

to traditional cement bricks (Table 6.7). By applying controlled force until the bio-bricks fractured, the machine measured their capacity to withstand pressure, which reflects the effectiveness of the bio-cementation process in binding sand particles. Higher compressive strength would indicate successful calcium carbonate precipitation and effective particle cohesion within the bio-bricks, confirming the potential of this biotechnological approach for construction applications.

The formula used to measure the strength:

Compressive Strength=Cross-Sectional Area/Load

Table 6.7: Compressive strength Analysis of bio-cement

Sr. No.	**Fungus Inoculated Cube**	**Load (kN)**	**Compressive strength**
1.	Control	0	0 MPa
2.	*Penicillium chrysogenum*	7.4	15.10 MPa
3.	S1(3)	6.2	12.60 MPA
4.	S4(9)	5.2	10.61 MPa
5.	S6(9)	5.1	10.40 MPa

6.8.2 Water absorption (Porosity testing)

The water absorption test of fungi bio-bricks evaluates the ability of these bio-composite materials to absorb and retain moisture, which is crucial in determining their durability and suitability for construction applications. In this test, bio-bricks are typically weighed before and after immersion in water for a specific period, such as 24 hrs. The increase in weight, represented as a percentage, indicates the amount of water absorbed. Lower water absorption rates suggest higher moisture resistance, contributing to the brick's stability, longevity, and structural integrity. Since fungi bio- bricks contain organic and inorganic components, such as fungi biomass and calcium

carbonate, the test can reveal insights into how these materials respond to water exposure. Optimal fungi bio-bricks should exhibit low water absorption, ensuring they can withstand environmental conditions without significant degradation (table 6.8)**.**

Formula: Water absorption Final Weight - Initial weight/final weight x 100

Table 6.8: Water absorption test

Sr. No.	**Fungus**	**Initial weight(g)**	**Final weight(g)**	**Percentage of absorption**
1	Control	450.0	0	0%
2	*Penicillium chrysogenum*	451.55	486.212	7.7%
3	S1(3)	452.190	490.083	6.9%
4	S4(9)	498.690	527.125	5.6%
5	S6(9)	481.560	550.821	12%

6.9 Instrumentation Analysis

6.9.1 ATR-FTIR (Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy)

In ATR-FTIR analysis, absorption bands represent specific vibrations of chemical bonds within the molecules.

Control

The absorption peak at 2975 cm^{-1} is due to C-H symmetric vibrations of saturated hydrocarbons. The peak at 1406 cm^{-1} is due to the H-O-H stretch. The presence of silica and other components such as acids and aliphatic esters in sand particles is confirmed by peaks at 1050 cm^{-1} and 775 cm^{-1} due to Si-O-Si and Si-O stretch (Li et al., 2017) (Figure 6.16).

MICP Process

The strong absorption bands in the range of 1600-1620 cm^{-1} and 1300-1390 cm^{-1} in all the samples (*Penicillium chrysogenum* (fig 6.17), S1(3) (Figure 6.18), S4(9) (Figure 6.17), S6(9) (Figure 6.19)) attribute to the amorphous phase of calcite and aragonite confirming the bio-cement formation due to fungus (Perez et al., 2020).

For bio-cement made with fungi, the absorption bands can be interpreted as follows:

- **3330-3340 cm^{-1} (O-H stretching vibrations):**

  The absorption band at 3338 cm^{-1} in ATR-FTIR typically corresponds to O-H stretching vibrations. This band is usually associated with hydrogen-bonded hydroxyl groups (–OH) or water molecules. The bio-cement contains hydrated minerals, such as hydrated calcium carbonate ($CaCO_3.H_2O$) or other hydrated salts, the O-H stretch could correspond to water molecules associated with these phases (Ye et al., 2023).

- **2980- 2990 cm^{-1} (C-H stretching vibrations):**

  This peak is likely attributed to the aliphatic C-H stretching vibrations. It suggests the presence of organic components, such as lipids, proteins, or polysaccharides produced by the fungus during the biomineralization process (Liang et al., 2022).

- **1614 cm^{-1} (C=O stretching or COO^- stretching):**

  This band can be assigned to the asymmetric stretching of carboxylate groups (COO^-) or C=O stretching vibrations in the amide I bands (proteins or peptides). In bio-cement, this could indicate the presence of fungi secretions, such as organic acids or proteins involved in the cementation process (Devgon et al., 2024).

- **1300-1400 cm^{-1} (COO^- symmetric stretching):**

  This band is typically associated with symmetric stretching vibrations of the carboxylate group (COO^-). It further supports the involvement of organic acids or polysaccharides produced by fungi in the bio-cement formation, contributing to the mineralization process.

- **1066 cm^{-1} (Si-O stretching vibrations):**

The prominent absorption band at 1066 cm^{-1} in the ATR-FTIR spectrum of bio-cement made with fungi is primarily associated with Si-O stretching vibrations, indicating the presence of silica (SiO_2) or silicate minerals. This suggests that silica-based materials are involved in the bio-cement formation process, likely due to the fungi activity promoting silica precipitation. Alternatively, this band may also represent C-O stretching in carbonate ions (CO_3^{2-}), reflecting the formation of calcium carbonate ($CaCO_3$), a key component of bio-cement. Thus, the peak at 1066 cm^{-1} signifies the involvement of both silica and carbonate phases in the bio-cement matrix (He et al., 2023).

- **770-780 cm^{-1} (Si-O or $CaCO_3$ vibrations):**

  This band may be attributed to Si-O stretching or bending vibrations, indicating the presence of silica-based minerals in the bio-cement (Ferral-Pérez, H., & Galicia-García, M. (2020)). Alternatively, it could correspond to the bending mode of carbonate ions ($CaCO_3$), which suggests calcium carbonate precipitation, a key component of bio-cement (Zhao et al., 2022).

  In summary, the absorption bands point to a combination of organic (fungi secretions like polysaccharides or proteins) and inorganic (calcium carbonate or silica) components in the bio-cement. These findings reflect the role of fungi in biomineralization, where organic compounds produced by the fungi facilitate the precipitation of minerals like calcium carbonate or silica, contributing to the formation of the bio-cement.

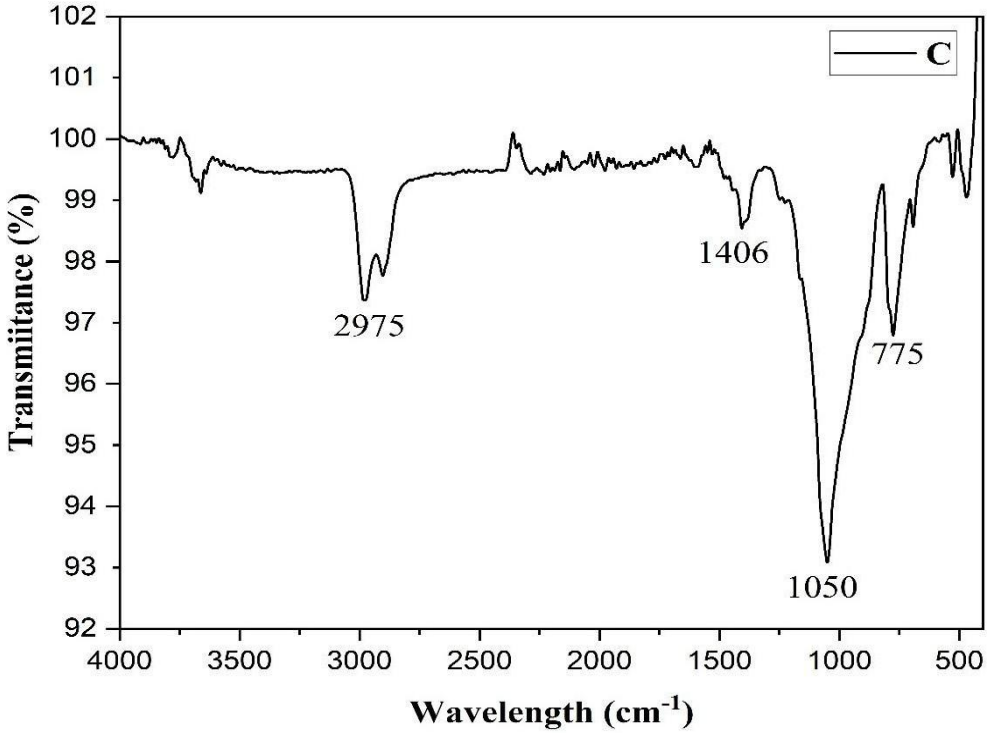


Figure 6.16: ATR-FTIR spectra of control

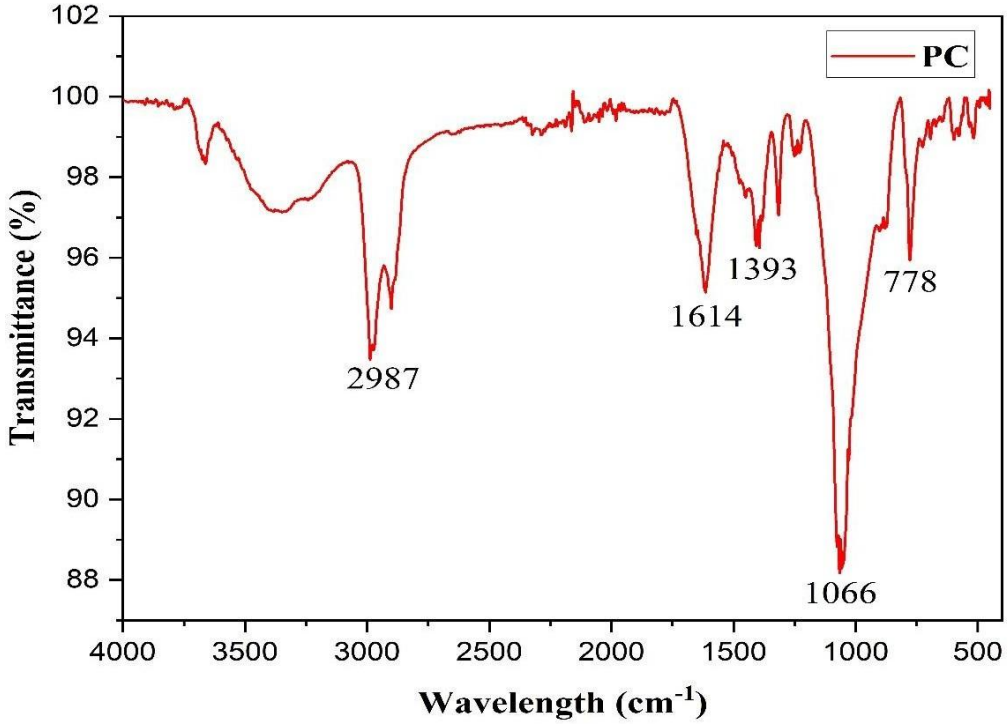


Figure 6.17: ATR-FTIR spectra of *Penicillium chrysogenum*

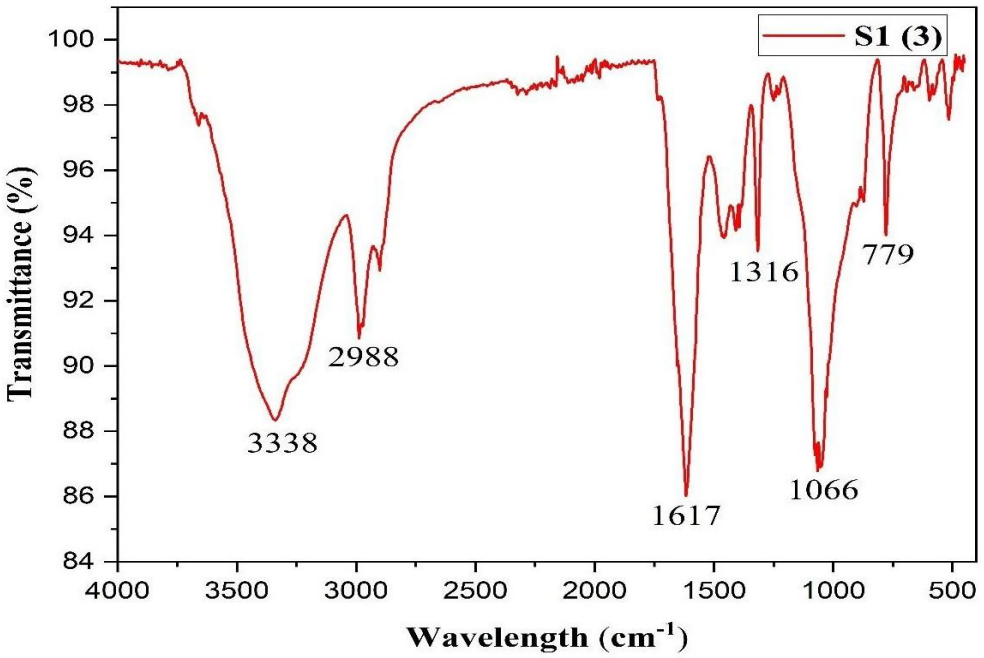


Figure 6.18: ATR-FTIR spectra of fungi isolate S1(3)

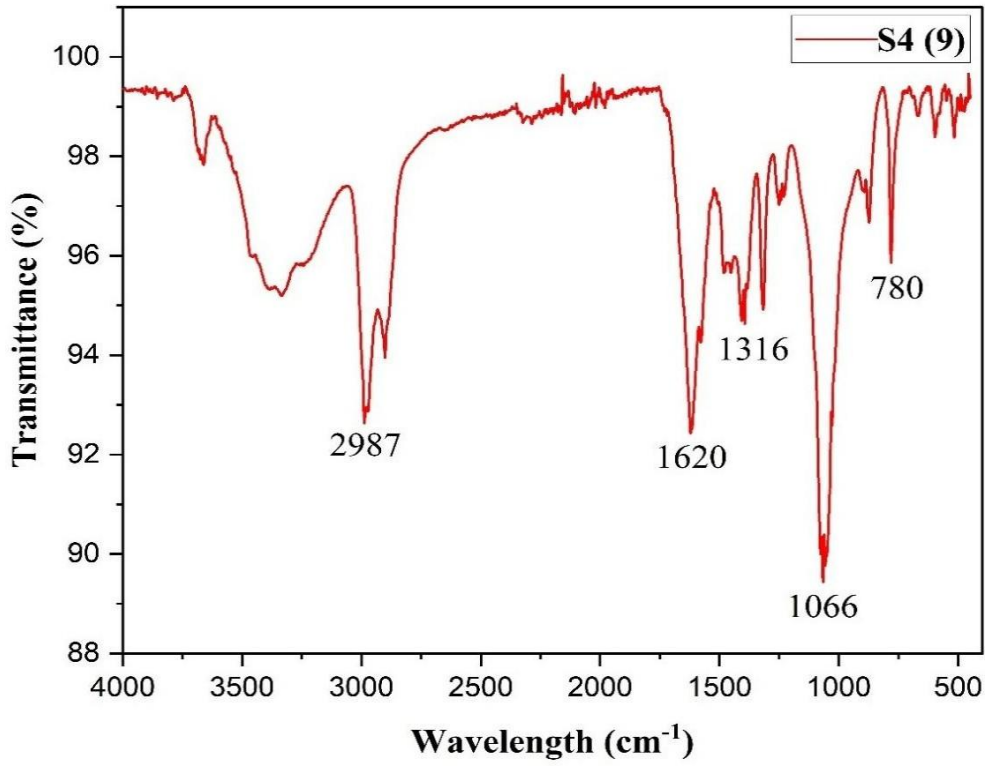


Figure 6.19: ATR-FTIR spectra of fungi isolate S4(9)

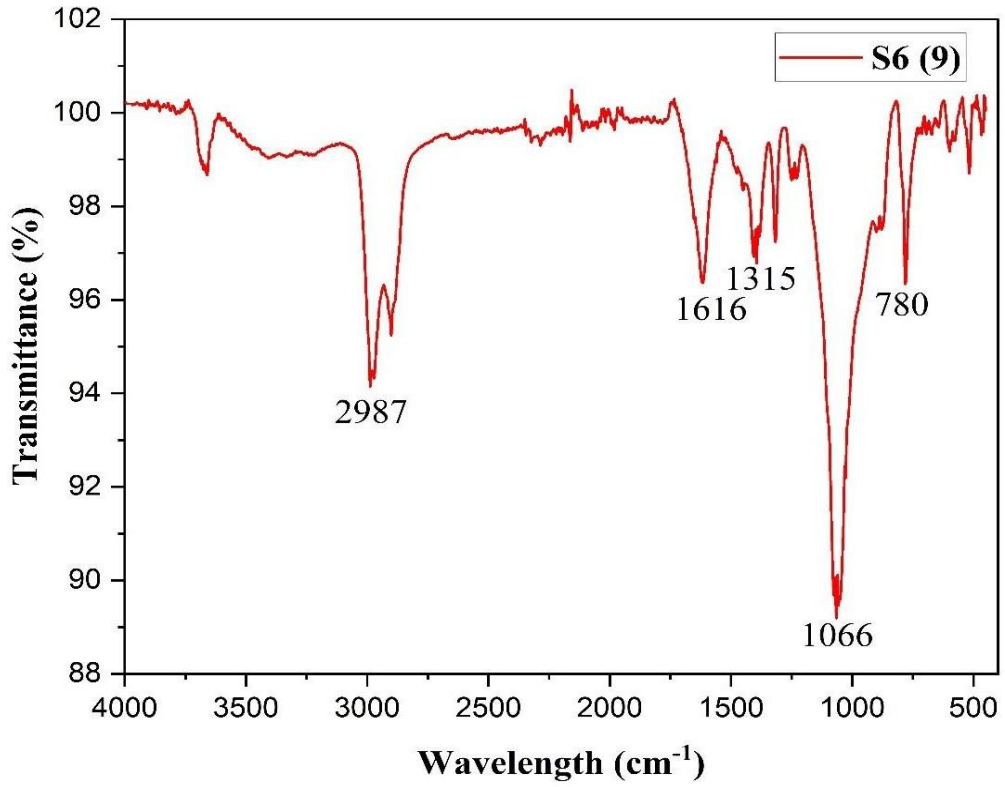


Figure 6.20: ATR-FTIR spectra of fungi isolate S6(9)

6.9.2 X-Ray Diffraction (XRD)

The prominent peaks at crystal planes (100), (011), (110), (200), and (203) were observed at 2θ values of 20.9°, 26.6°, 36.6°, 42.5°, and 68.2° in the sand sample (Control) arise from the diffraction of X-rays by the crystalline structures present in the sample as seen in the Figure 6.21. These peaks indicate the presence of mineral components commonly found in soil, such as quartz, feldspar, and other silicate minerals. The most significant peak at 26.6° corresponds to the (011) plane of quartz, a major component of sand, reflecting the crystalline structure of SiO_2. Quartz, being highly abundant in sand, typically exhibits strong diffraction patterns. The other peaks at lower and higher angles are likely due to additional phases or polymorphs of silicates, such as feldspar, which contribute to the diffraction pattern from planes like (100), (110), and (200). These planes reflect the internal atomic arrangement of these minerals, and their specific 2θ values correspond to the spacing between atomic layers, following

Bragg's Law. The presence and intensity of these peaks are characteristic of the mineral composition and crystallinity of the soil components in the sand (Devgon et al., 2024).

The ureolytic pathway of biomineralization, mediated by fungi, leads to the formation of calcium carbonate in the form of bio-cement. This process results in the precipitation of calcite and vaterite, two crystalline polymorphs of calcium carbonate, which can be detected through X-ray diffraction (XRD). In *Penicillium chrysogenum* (Figure 6.22), the diffraction peaks are observed at crystal planes (100), (011), (200), and (112) at 2θ values 20.9, 26.6, 42.5, and 50.2 respectively. For S1(3) the XRD diffractogram, prominent peaks are observed at 2θ values of 20.9°, 26.6°, 39.5°, and 50.6°, corresponding to the diffraction from crystal planes (100), (001), (102), and (003), respectively Figure (6.23) (Qian et al., 2018; Debnath et al., 2023). These peaks are indicative of the specific lattice planes within the calcite and vaterite structures, with the (001) and (003) planes being characteristic of the rhombohedral crystal system of calcite, while the (100) and (102) planes may correspond to vaterite, a less stable but commonly formed polymorph in biological systems. The observed peaks confirm the mineralization of calcium carbonate facilitated by fungi urease activity, where urea hydrolysis leads to increased pH and carbonate ion production, enabling the crystallization of calcite and vaterite, contributing to bio-cement formation (Khan et al., 2023).

In samples, S4(9) (Figure 6.24) and S6(9) (Figure 6.25), the observed XRD peaks at crystal planes (100), (011), and (200) for S4(9) at 2θ values of 20.917°, 26.8°, and 42.8°, reflect the formation of calcium carbonate minerals, specifically calcite, as part of the bio-cementation process facilitated by different fungi species. These fungi catalyze the ureolytic pathway, where urea is hydrolyzed to produce ammonium and carbonate ions, leading to a localized pH increase and subsequent precipitation of calcium carbonate (Zhao et al., 2022). The distinct peaks correspond to the crystallographic planes of calcite, with the (100) and (011) planes being particularly indicative of calcite formation. The presence of similar peaks in both S4 (9) and S6 (9) suggests that both fungi species promote calcite precipitation, but the variation in the occurrence of the (200) plane in S4(9) may indicate differences in the crystallization environment or fungi activity, leading to more pronounced calcite formation in specific orientations (Luo et

al., 2018). These structural differences could arise due to factors such as the metabolic byproducts of the fungi, the rate of urea hydrolysis, or the availability of calcium ions, all of which influence the nucleation and growth of calcite crystals during bio-cement formation. The results demonstrate that while all fungi species facilitate bio-cementation, the specific crystal growth patterns and mineral phases formed can vary depending on fungi metabolism and environmental conditions.

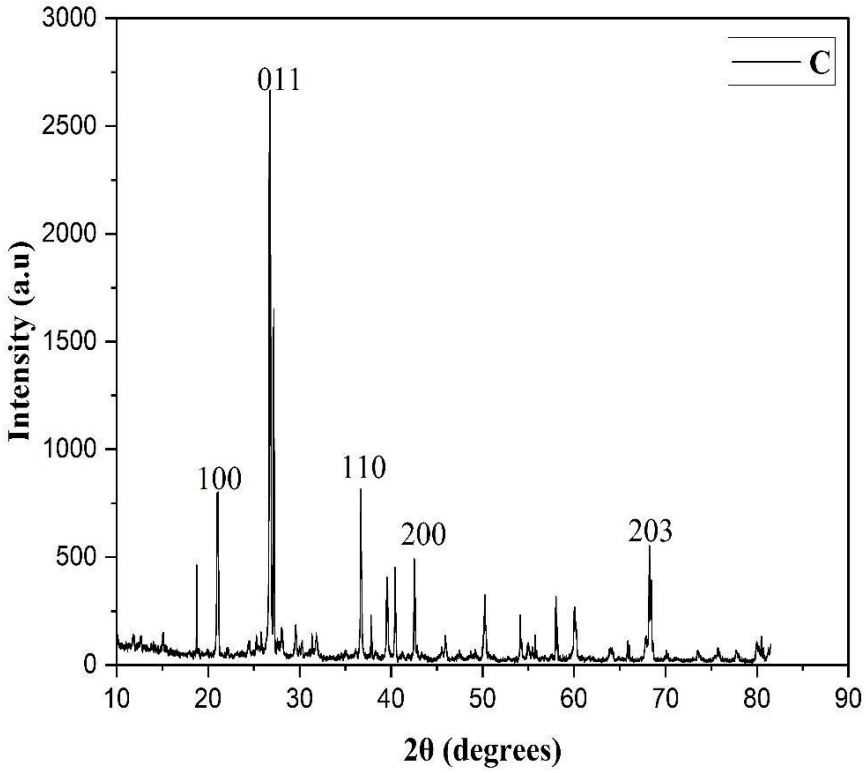


Figure 6.21: XRD diffractogram of Control (C)

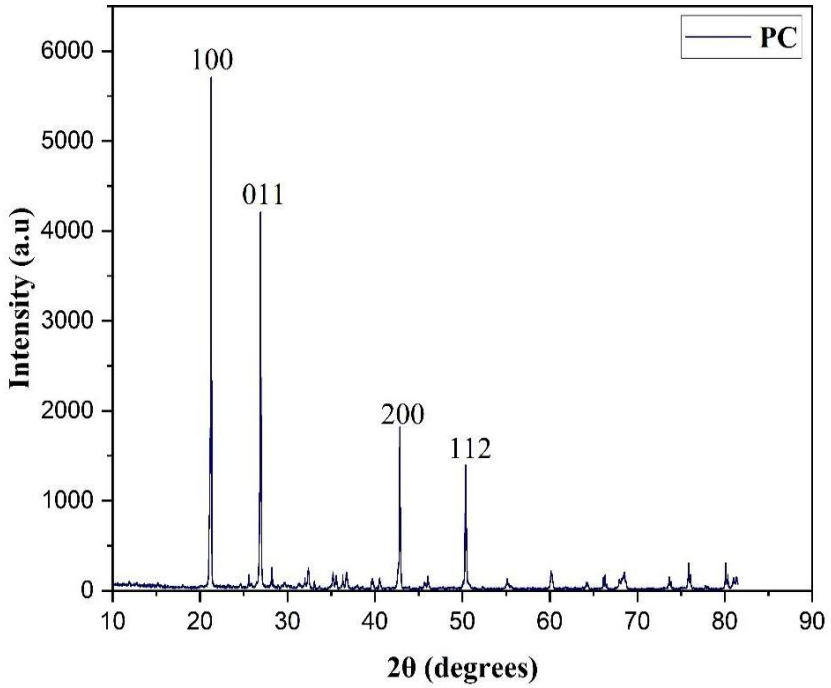


Figure 6.22: XRD diffractogram of *Penicillium chrysogenum*

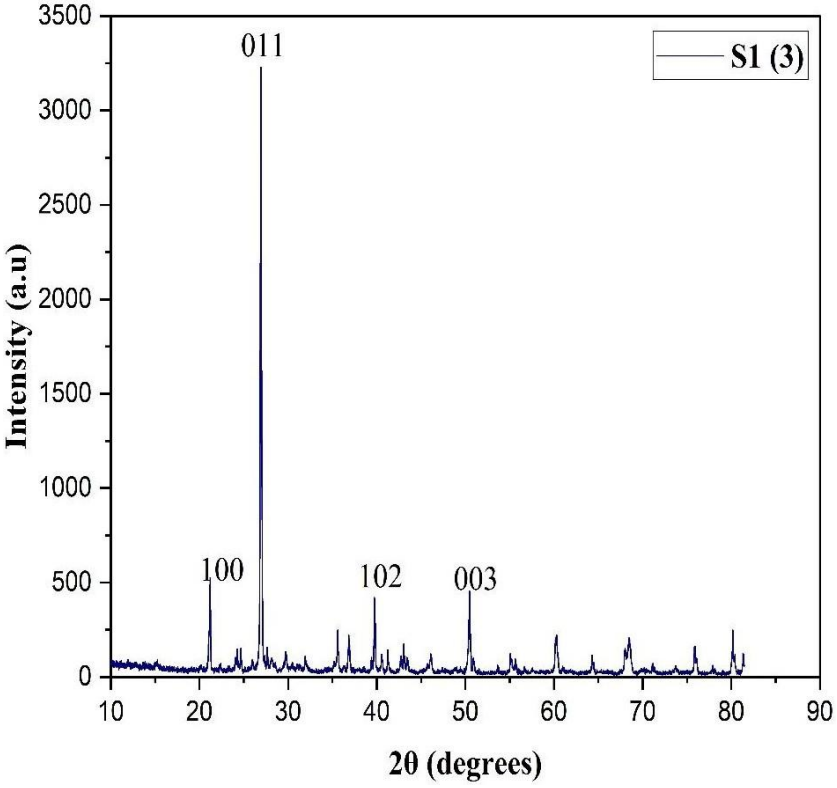


Figure 6.23: XRD diffractogram of fungi isolate S1(3)

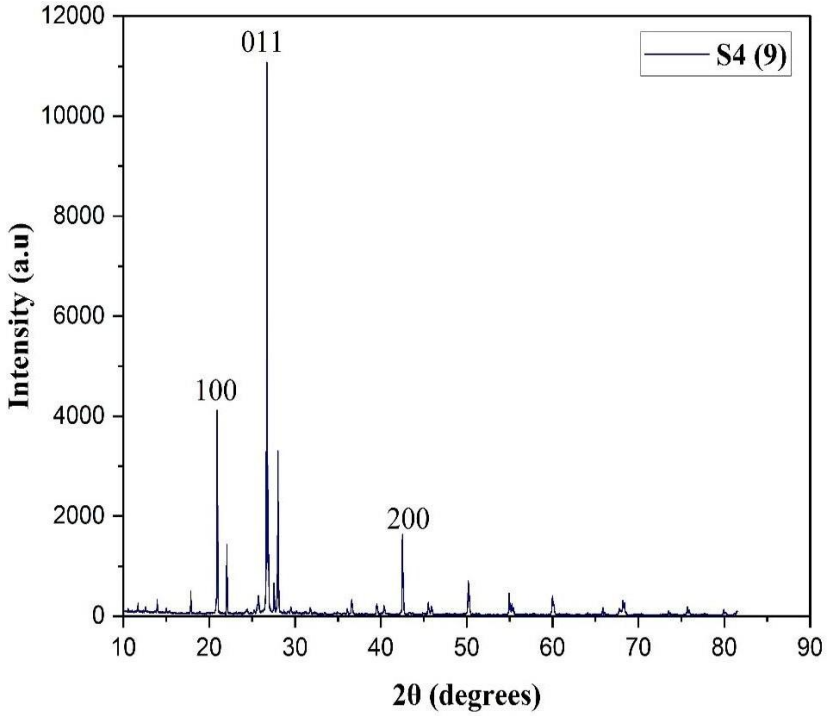


Figure 6.24: XRD diffractogram of fungi isolate S4(9)

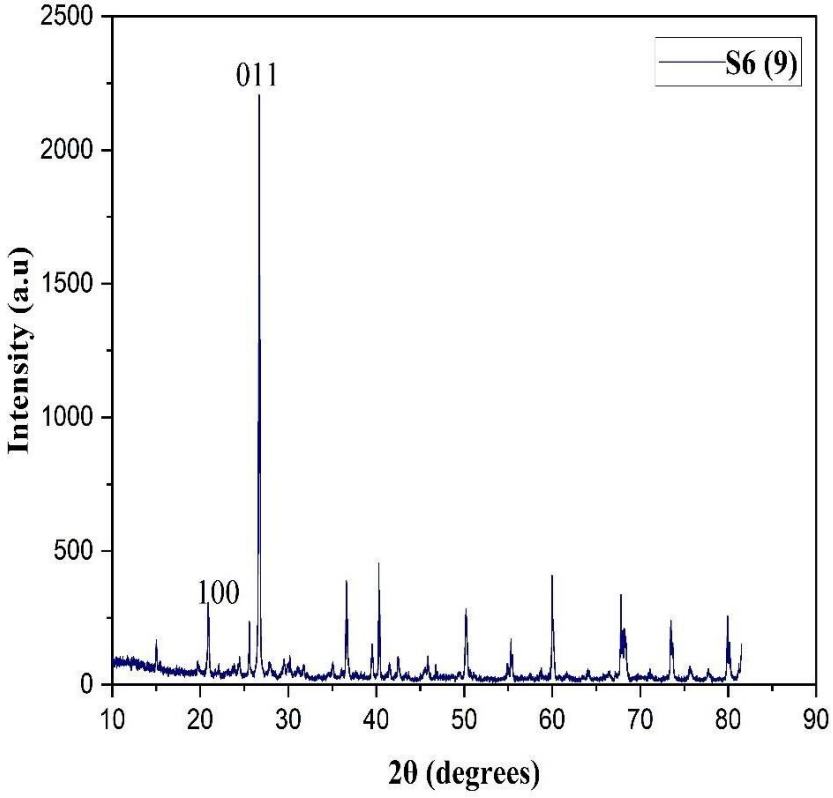


Figure 6.25: XRD diffractogram of fungi isolate S6(9).

6.9.3 Field Emission Scanning Electron (FE-SEM)

The FESEM analysis of bio-cement bricks revealed a robust microstructure characterized by uniform pore distribution, fungi filament integration, and calcium-rich areas, suggesting effective biomineralization. The detailed surface morphology showed porous regions intertwined with fungi mycelium, enhancing bonding within the matrix and contributing to structural integrity. Elemental mapping confirmed calcium, silicon, and oxygen presence, highlighting the role of fungi activity in stabilizing the cement matrix through calcium carbonate precipitation. Compared to non-inoculated controls, bio-cement bricks exhibited more cohesive internal structures, likely translating to improved compressive strength. These results underscore the potential of bio-cement as a sustainable, durable alternative for construction materials (Khan et al., 2023; Devgon et al., 2024).

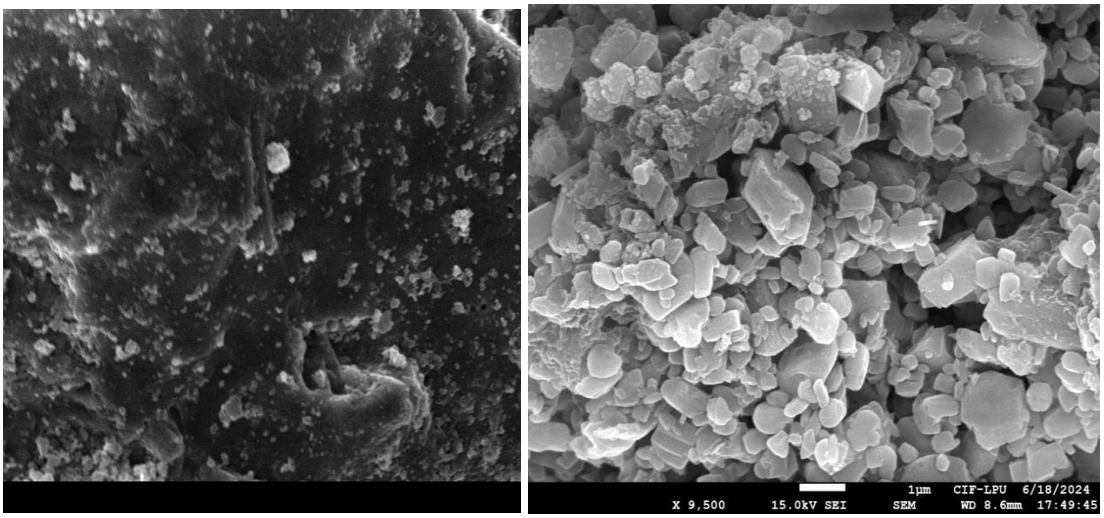
Qian et al. (2017) analyzed that calcium carbonate crystals on the surface of the mycelial nucleus of *P. chrysogenum* CS1 had a size of 200 to 600 nm, which is much smaller than the crystals visible under the light microscope (1 to 10 m). These precipitates formed pleomorphic, thick shells covering the mycelia, arranged in rows and tightly connected. The element types and weight distributions of various elements produced by *P. chrysogenum* CS1 during carbonate precipitation varied considerably, as shown in the EDS spectra. These results demonstrate the presence of C, O, Ca, and trace amounts of Cl in the biomineralization products that developed on the mycelia during MICP. Menon et al. (2019) studied the crystallinity of calcium precipitates by *Aspergillus nidulans*, the precipitates formed were higher, as evidenced by the uniform shape of the particles and the well-defined crystalline faces. The mineral crystals exhibited a variety of crystalline morphologies, from a single pure bulk crystal to stacked platelet-shaped crystals. In addition, the crystals showed the presence of fungi as they had numerous cylindrical holes that most likely formed in an area where fungi were present. The holes also showed that fungi filaments were nucleation sites throughout the biomineralization process (Menon et al., 2019).

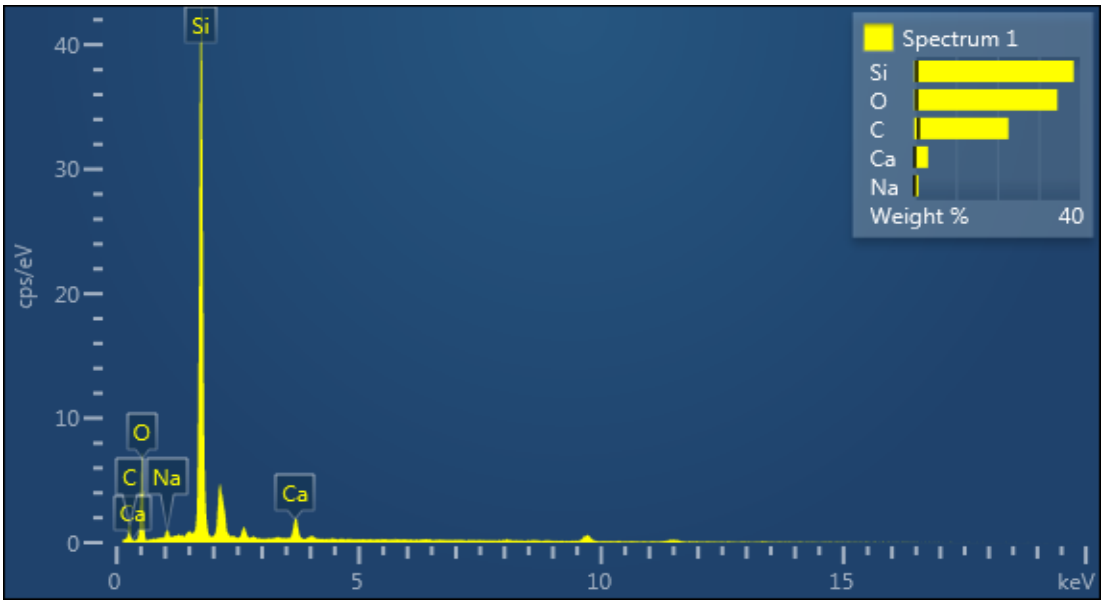
Figure 6.26 represents the Control sample (C) where SEM-EDS of soil is done. The morphology is revealed in SEM images showing different geometries of sand i.e., rounded, angular and rectangle shaped. The EDS showed that the majority element

present is silica and oxygen concluding the control is quartz sand (Adewunmi et al., 2020).

Figures 6.27, 6.28, 6.29, and 6.30 depicts the SEM analysis of calcite precipitation, revealing the presence of larger mineral crystals within fungi isolates *Penicillium chrysogenum*, S1 (3), S4 (9), and S6 (9) respectively. The crystals are evidence that fungi isolates are involved in forming calcite precipitation. The accumulation of calcite near fungi hyphae indicates a nucleation site for mineralization. The crystals formed showed planner and irrgeular shaped geometry and are formed one above another showing precipitation. EDS analyzed the confirmation of calcite that showed the crystal formed were elements of Ca, C, and O that closely resembled the atomic percentage of $CaCO_3$. Silica percentage is reduced in the bio-cementation process and the concentration of elements such as Ca, C and O increases.

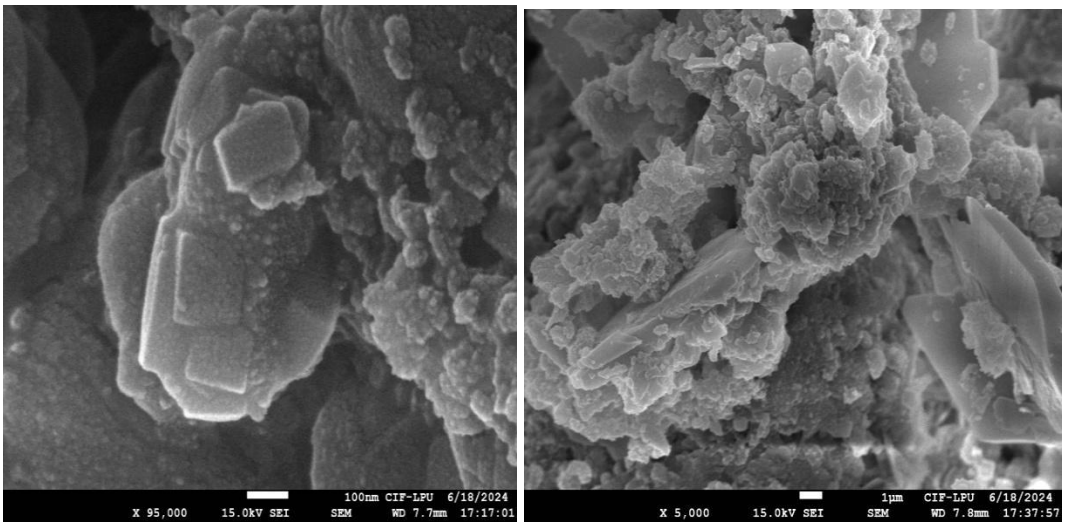
These crystals serve as tangible evidence of the active involvement of fungi isolates in the formation of calcite precipitation (Burforf et al., 2006). The localized accumulation of calcite near fungi hyphae suggests the existence of nucleation sites for mineralization (Fomina et al., 2006; Ye et al., 2023).

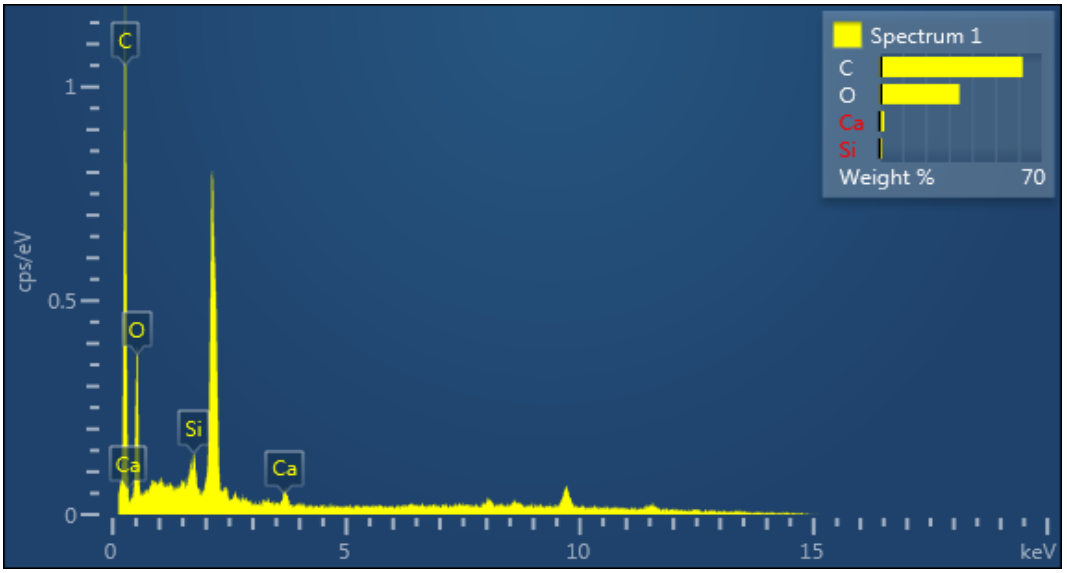




Spectrum 1	Atomic %
C	34.12
O	38.93
Na	0.76
Si	24.71
Ca	1.48
Total	100.00

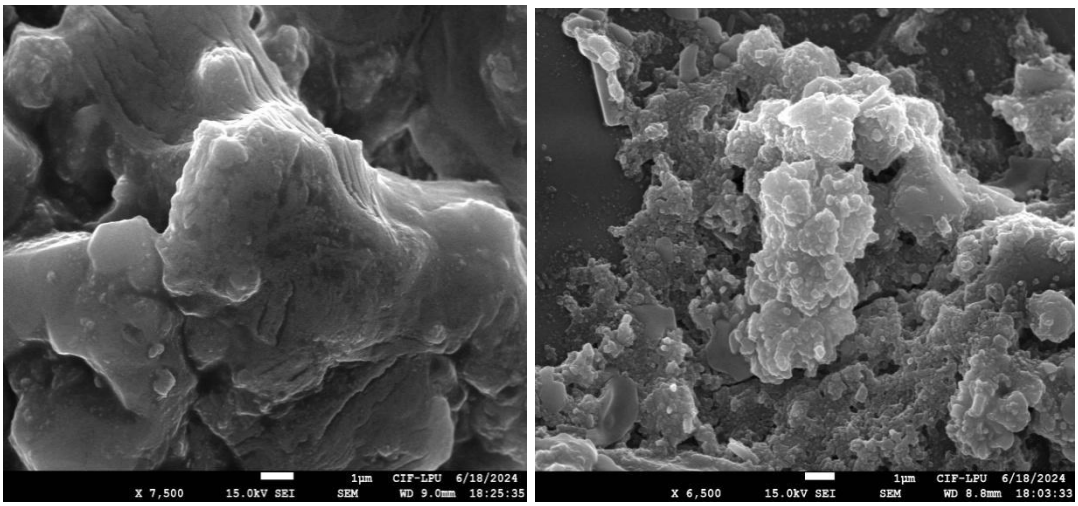
Figure 6.26: SEM and EDS spectra of control (C)

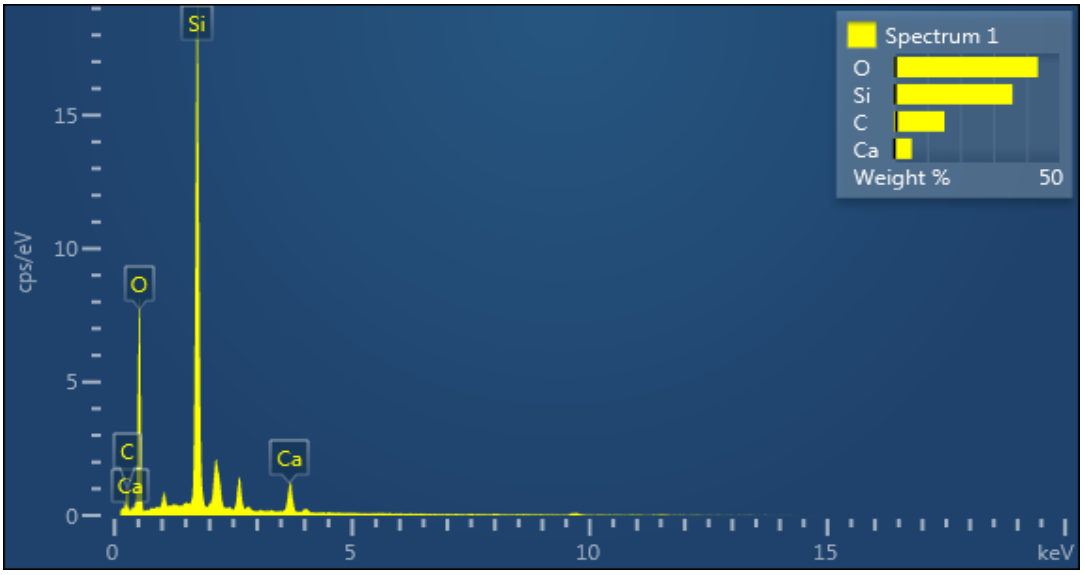




Spectrum 1	Atomic %
C	69.39
O	29.20
Si	0.66
Ca	0.75
Total	100.00

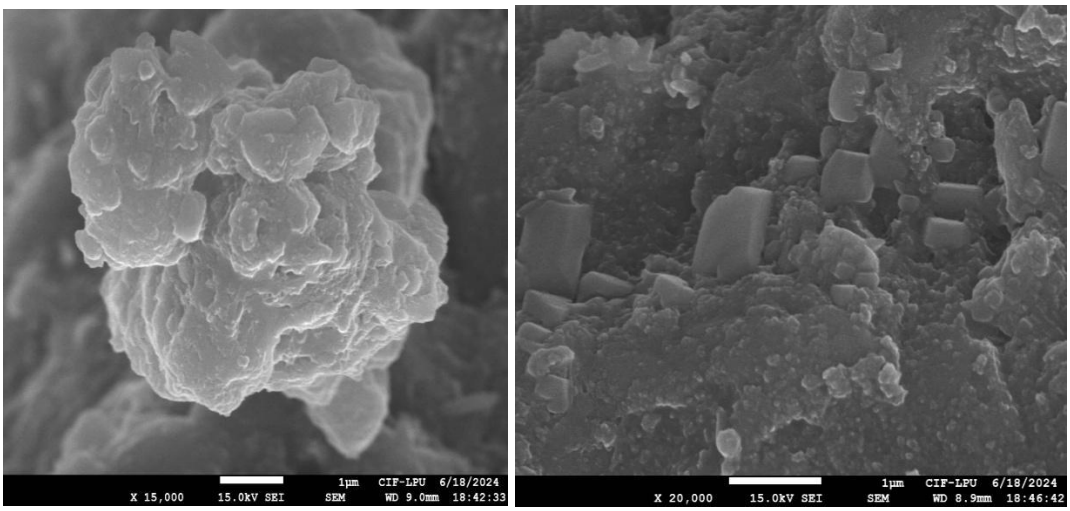
Figure 6.27: SEM and EDS spectra of calcite precipitation in *Penicillium chrysogenum*

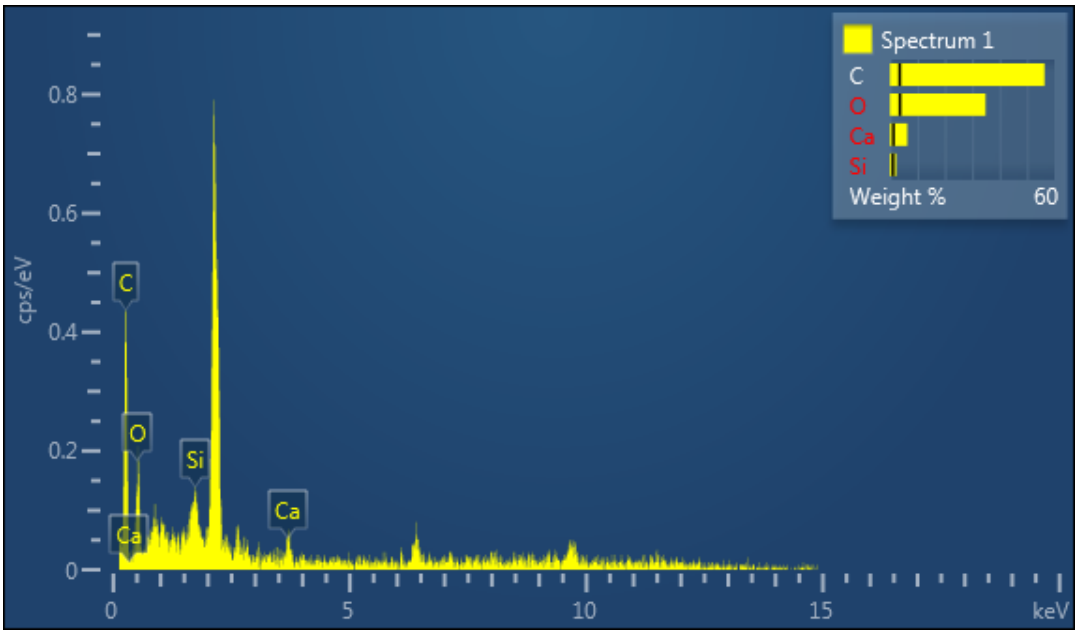




Spectrum 1	Atomic %
C	23.52
O	50.36
Si	23.63
Ca	2.49
Total	100.00

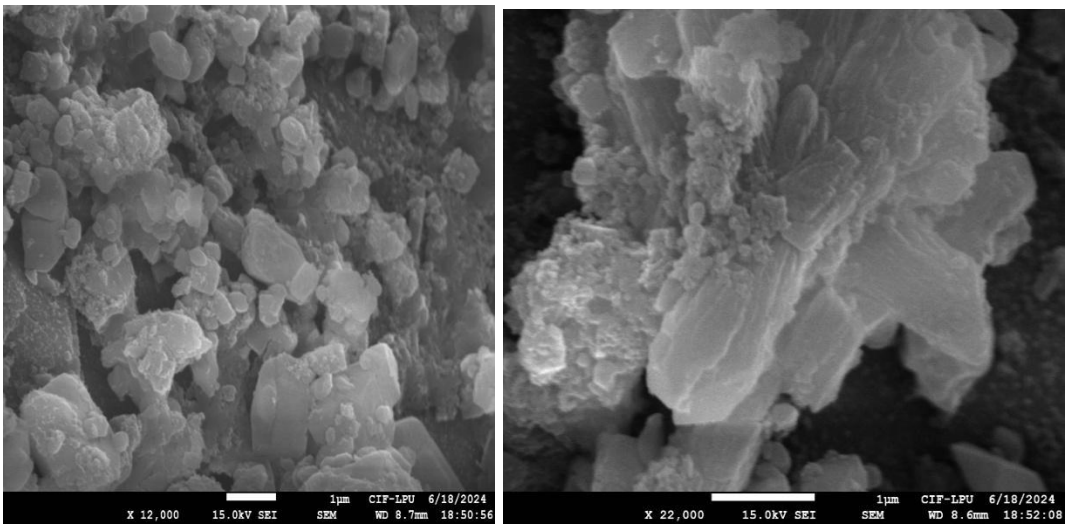
Figure 6.28: SEM and EDS spectra of calcite precipitation in fungi isolate S1 (3).

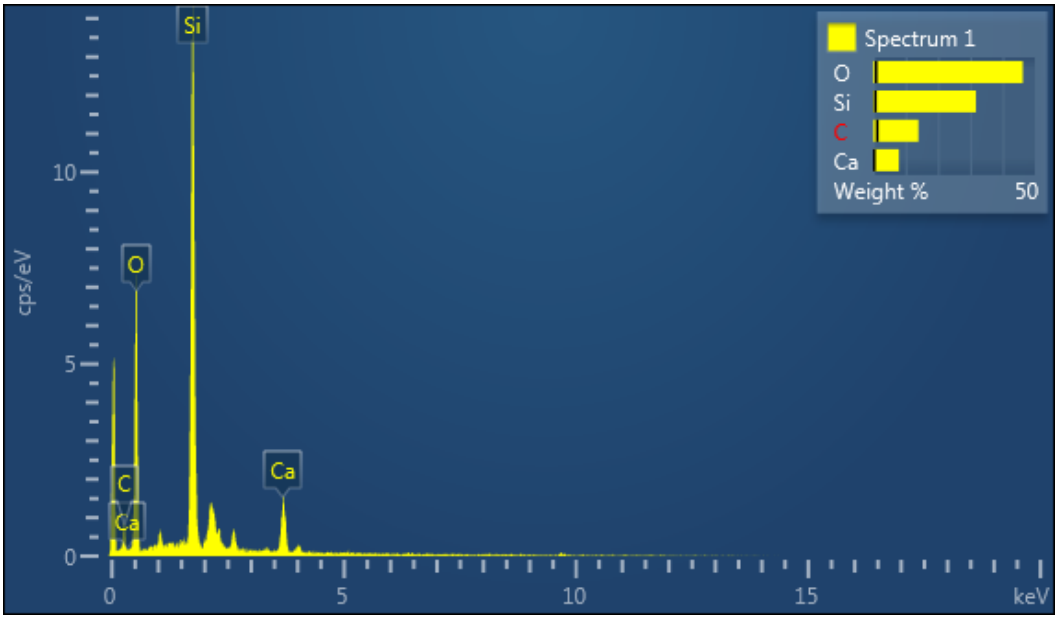




Spectrum 1	Atomic %
C	65.98
O	30.59
Si	1.19
Ca	2.24
Total	100.00

Figure 6.29: SEM and EDS spectra of calcite precipitation in fungi isolate S4 (9).





Spectrum 1	Atomic %
C	21.64
O	53.68
Si	21.01
Ca	3.67
Total	100.00

Figure 6.30: SEM and EDS spectra of calcite precipitation in fungi isolate S6(9).

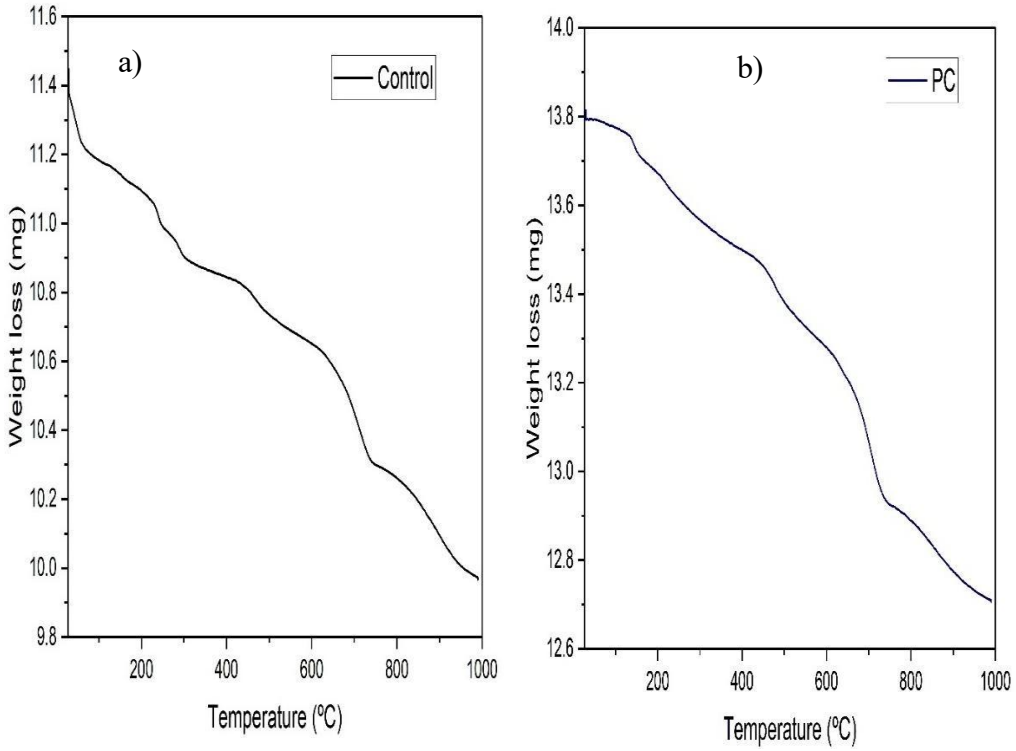
6.9.4 Thermogravimetric Analysis (TGA)

Thermal degradation in fungi-mediated microbial-induced calcium carbonate precipitation (Fungi-MICP) involves the breakdown of fungi biomass and calcium carbonate ($CaCO_3$) precipitates at elevated temperatures. Fungi proteins denature and cell walls decompose above 60-200°C, disrupting metabolic activity essential for $CaCO_3$ formation. The organic matrix secreted by fungi degrades, weakening crystal stability. $CaCO_3$ begins to decompose into calcium oxide (CaO) and CO_2 around 600-900°C, altering its morphology and reducing MICP efficiency. This degradation limits the process's efficacy, impacting applications where both fungi activity and $CaCO_3$ stability are crucial (Liang et al., 2022, Kang et al., 2022).

The thermalgravimetric analysis of control, bio-cement prepared from *Penicillium chrysogenum*, S1(3), S4(9), and S6(9) was done to check the thermal stability of the product. In control (C) (Figure 6.31 a), there is 13.66% total thermal degradation due to the decomposition of organic impurities, moisture loss, or the breakdown of carbonates, typically between 200-900°C. Pure silica sand (SiO_2) remains stable at high temperatures (Choi et al., 2017). In Bio-cement- *Penicillium chrysogenum* (Figure 6.31b), the first stage of degradation begins at 123.43°C at which the sample loss only 1% of its weight. The second stage is at 436.90°C where the weight loss percent is nearby 4%. In the last stage, there is a total 9% weight loss at 690°C and the bio-cement displays stable chemistry in its structure withstanding high temperatures with minimum weight loss (Yu et al., 2022).

In the Bio-cement (S1(3)) (fig 6.31c), the first stage of degradation starts only after 400°C where only 5% of the weight is lost and the second stage starts around 650°C due to the breakdown of $CaCO_3$ into Ca0 and CO_2 which further leads to total 13% weight loss of the bio-cement. The thermal degradation of S4 (9) starts at 108°C where fungi organic components degrade and further is followed by second and third stages of degradation at 435°C and 613°C resulting in only 11% thermal degradation (Figure 6.31d). Only 8% of the total weight is lost in sample S6 (9), exhibiting remarkable thermal stability at higher temperatures (Figure 6.31e) (Ditta et al., 2024).

The bio-cement prepared by fungi-MICP shows less thermal degradation based on thermogravimetric analysis (TGA), which indicates higher thermal stability. TGA measures the weight loss of materials as a function of temperature, so less thermal degradation suggests that both the fungi biomass and calcium carbonate precipitates are more resistant to breakdown at elevated temperatures. This implies that the bio-cement has stronger thermal stability, with the reduced decomposition of calcium carbonate into calcium oxide (CaO) and better preservation of the fungi organic matrix, enhancing the long-term durability of the bio-cement under thermal stress (Khushnood et al., 2022; Mokhtar et al., 2021).



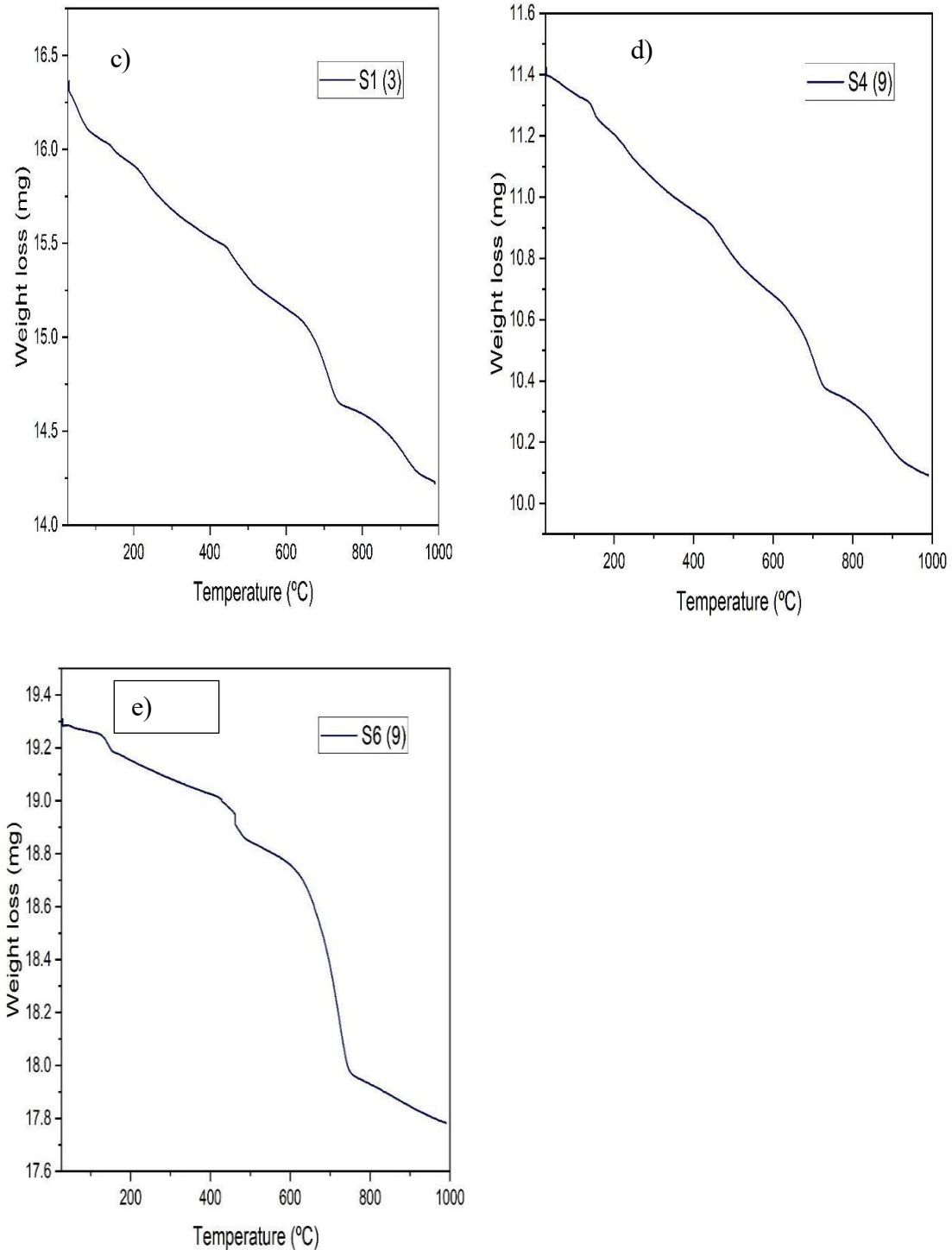


Figure 6.31: Thermogravimetric Analysis of a) Control (C), b) (*Penicillium chrysogenum*), c) (S1(3)), d (S4(9)), and e) (S6(9)).

6.10 Crack filling

In the wall crack-filling experiment using fungi-based bio-cement, visible results demonstrated the effectiveness of microbial-induced calcite precipitation (MICP) in sealing cracks shown in figure 6.32. The process involved introducing a fungi spore suspension with a nutrient solution containing calcium chloride and urea into pre-formed cracks on the wall surface. Over a period of 28 days, the fungi actively precipitated calcium carbonate within the cracks, visibly reducing their size and improving surface continuity. The calcium carbonate deposits adhered well to the wall substrate, forming a sturdy, natural filler that seamlessly bonded to the existing material. This self-filling capability of the fungi bio-cement exhibited promising results for structural repair, offering a sustainable and cost-effective alternative to conventional crack-filling materials. The results indicate that fungi bio-cement prepared by S1(3) has substantial potential for use in construction and restoration applications.

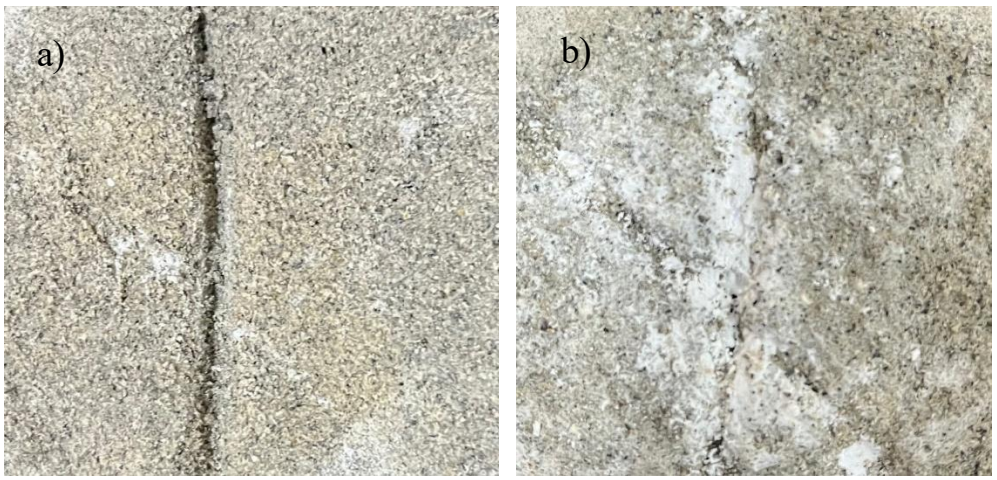


Figure 6.32: Crack-filling by fungi bio-cement S1(3) a) crack b) after 28 days

6.11 Genetic Identification (ITS)

The gel electrophoresis analysis of fungal DNA samples showed successful PCR amplification, with clear bands observed at the expected size (~600 bp). Fungal DNA Sample S1(3), S4(9), and S6(9) each displayed sharp bands around 600 bp, confirming proper amplification. The uniformity and clarity of these bands indicate high-quality PCR products with no signs of non-specific amplification or degradation. These results align with the typical size of internal transcribed spacer (ITS) region amplification used for fungal DNA identification, as shown in Figure 6.33.

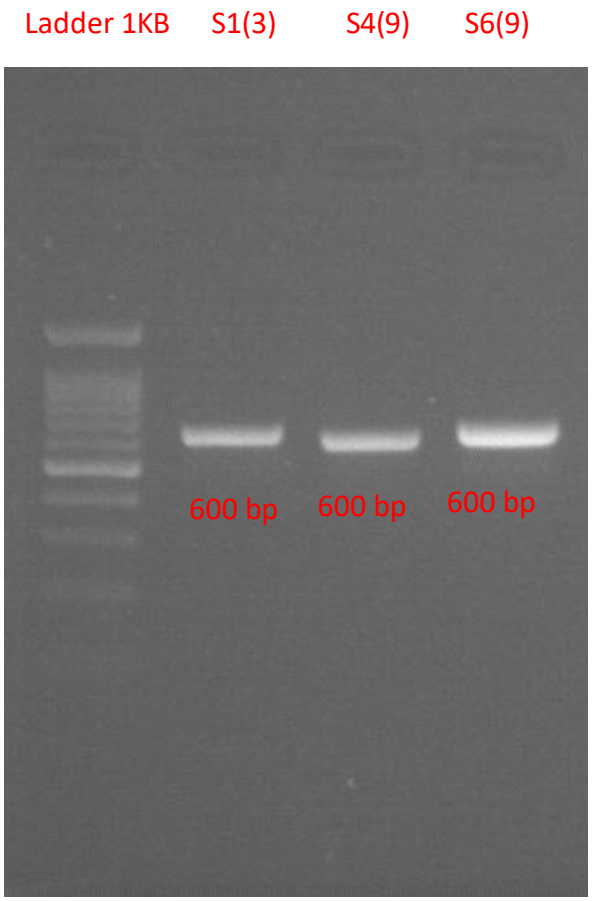


Figure 6.33: PCR- Gel electrophoresis of Fungi isolates

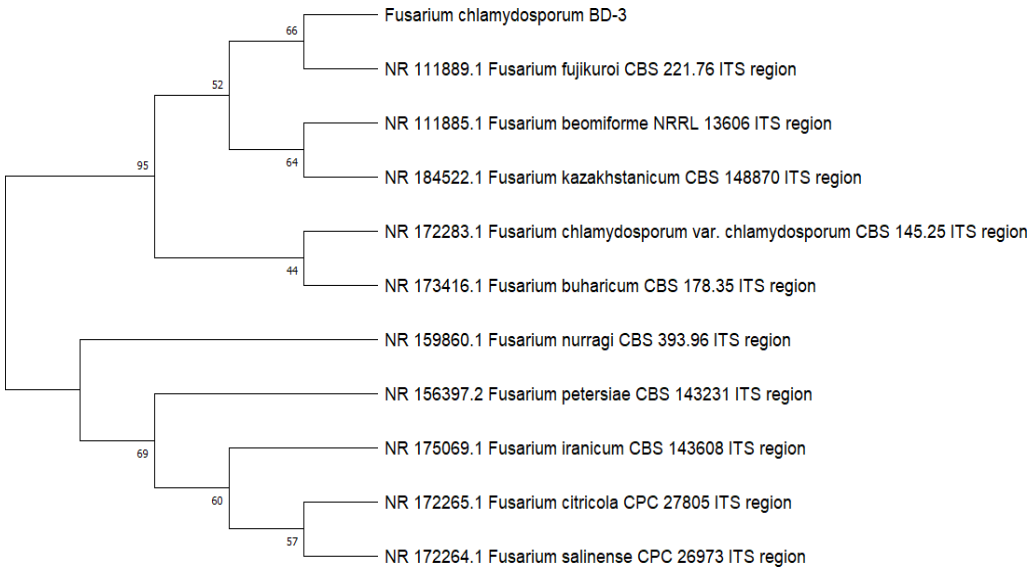


Figure 6.34: Phylogenetic analysis by Neighbour joining method for isolating *Fusarium chlamydosporum* BD-3.

The phylogenetic analysis of the sample labeled S1(3) identified it as *Fusarium chlamydosporum* based on nucleotide homology and phylogenetic comparisons shown in figure 6.34. These results were confirmed using ITS microbial screening genetic typing, with software tools considering the E value, which indicates the significance of matches based on the lowest scores. The evolutionary relationships were inferred using the Neighbor-Joining method as described by Saitou and Nei (1987). The analysis included a bootstrap test with 1,000 replicates to assess the reliability of the inferred tree. Branches reproduced in fewer than 50% of bootstrap replicates were collapsed, and the percentage of replicate trees in which associated taxa clustered is indicated next to the branches. The evolutionary distances were calculated using 11 nucleotide sequences, and ambiguous positions were removed using the pairwise deletion option, resulting in a final dataset of 627 positions. All evolutionary analyses were conducted using the MEGA11 software as described by Tamura et al. (2021). The isolate has been submitted to NCBI under the sample ID PQ479941 and has been named *Fusarium chlamydosporum* BD-3.

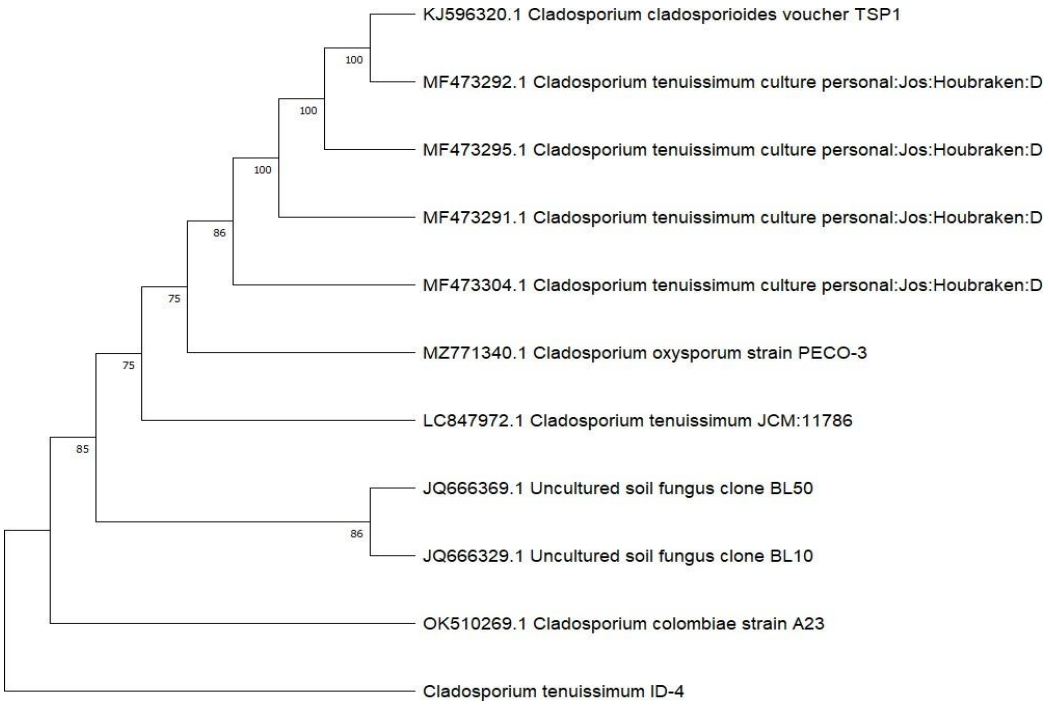


Figure 6.35: Phylogenetic analysis by Neighbour joining method for isolating *Cladosporium tenuissium ID-4*.

The phylogenetic analysis of the sample labeled as S4(9) identified it as *Cladosporium tenuissium* based on nucleotide homology and phylogenetic comparisons shown in figure 6.35. These results were confirmed using ITS microbial screening genetic typing, with software tools considering the E value, which indicates the significance of matches based on the lowest scores. The evolutionary relationships were inferred using the Neighbor-Joining method as described by Saitou and Nei (1987). The analysis included a bootstrap test with 1,000 replicates to assess the reliability of the inferred tree. Branches reproduced in fewer than 50% of bootstrap replicates were collapsed, and the percentage of replicate trees in which associated taxa clustered is indicated next to the branches. The evolutionary distances were calculated using the Jukes-Cantor method, which measures base substitutions per site. This analysis involved 11 nucleotide sequences, and ambiguous positions were removed using the pairwise deletion option, resulting in a final dataset of 638 positions. All evolutionary analyses were conducted

using the MEGA11 software as described by Tamura et al. (2021). The isolate has been submitted to NCBI under the sample PQ882141 and has been named *Cladosporium tenuissium ID-4*

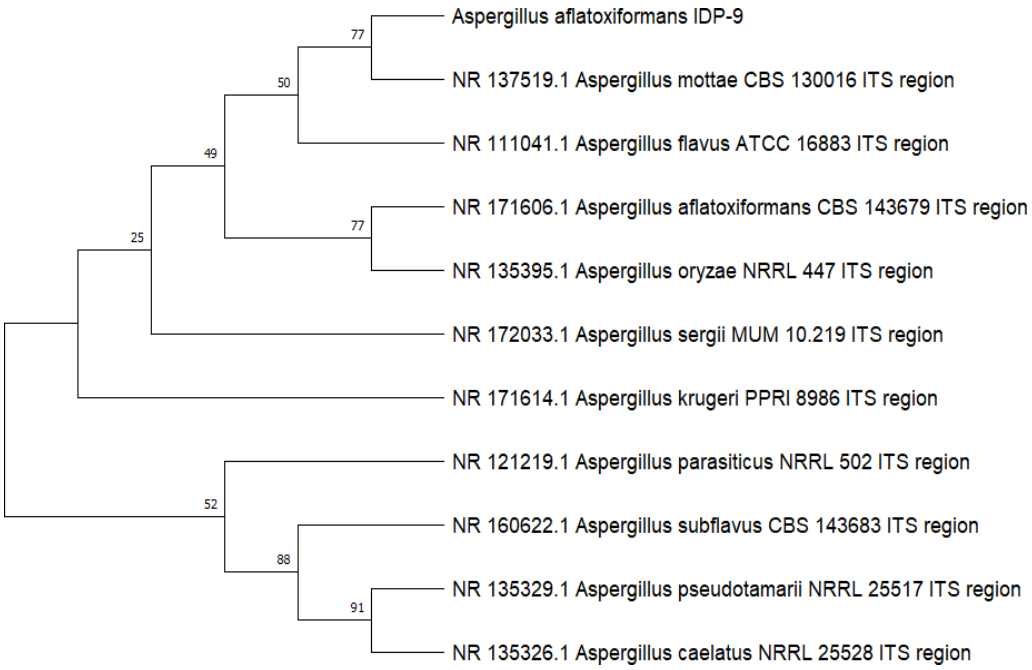


Figure 6.36: Phylogenetic analysis by Neighbour joining method for isolating *Aspergillus aflatoxiformans* IDP-9

The phylogenetic analysis of the sample labelled as S6(9) identified it as *Aspergillus aflatoxiformans* based on nucleotide homology and phylogenetic comparisons shown in figure 6.36. These results were confirmed using ITS microbial screening genetic typing, with software tools considering the E value, which indicates the significance of matches based on the lowest scores. The evolutionary relationships were inferred using the Neighbor-Joining method as described by Saitou and Nei (1987). The analysis included a bootstrap test with 1,000 replicates to assess the reliability of the inferred tree. Branches reproduced in fewer than 50% of bootstrap replicates were collapsed, and the percentage of replicate trees in which associated taxa clustered is indicated next to the branches. The evolutionary distances were calculated using the Jukes-Cantor method, which measures base substitutions per site. This analysis involved 11 nucleotide sequences, and ambiguous positions were removed using the pairwise

deletion option, resulting in a final dataset of 638 positions. All evolutionary analyses were conducted using the MEGA11 software as described by (Tamura et al., 2021). The isolate has been submitted to NCBI under the sample ID PQ479936 and has been named *Aspergillus aflatoxiformans* IDP-9.

CHAPTER 7

CONCLUSION AND SUMMARY

This study successfully isolated 46 fungal strains from alkaline soils in Punjab to explore their potential in microbial-induced calcite precipitation (MICP) for the production of bio-cement. The isolates underwent extensive qualitative and quantitative analyses to identify the most suitable candidates for biocement production. Among these, isolates S4(1), S4(9), S1(18), S6(9), and S1(3) exhibited significantly high urease production, making them ideal for applications requiring rapid and substantial calcite precipitation. The urease-positive fungi selected for their high enzymatic activity played a crucial role in generating carbonate ions essential for calcium carbonate formation. The calcium precipitation assay results demonstrated that isolates S1(3), S4(9), S1(18), S2(7), S1(28), and S6(9) had the lowest average burette values, measured as 2.3379±0.57, 2.7044±0.27, 3.3398±0.57, 4.0746±0.11, 7.7056±0.57 µg/mL, respectively. These values indicated strong calcite precipitation capabilities, effectively utilizing calcium ions to form stable calcium carbonate. In comparison, *Penicillium chrysogenum* (PC) demonstrated a burette value of 4.4628±0.33 µg/mL. The results suggested that the selected fungal isolates are prime candidates for biocementation, as they consistently precipitated calcium, forming structurally sound matrices. The statistical analysis of the urease assay and calcium precipitation assay using one-way ANOVA confirmed significant differences among the experimental groups, supporting the superior performance of the identified fungal isolates.

For the fungal growth medium, rice straw was collected and chemically pretreated to reduce lignin content and increase the accessibility of cellulose and hemicellulose. The biochemical characterization of pretreated rice straw included the Molisch test, which confirmed the presence of carbohydrates by forming a characteristic purple ring. The DNS assay quantified sugar release after chemical treatments: 2% NaOH yielded 0.189 µg/mL sugar, 4% NaOH yielded 0.359 µg/mL, 2% H_2SO_4 yielded 5.99 µg/mL, and 4% H_2SO_4 yielded 3.13 µg/mL. These results indicated that sulfuric acid treatments, particularly with 2% H_2SO_4, were most effective in releasing sugars for fungal growth. FTIR and XRD analyses confirmed the delignification of rice straw, validating its suitability as a fungal growth substrate. The optimization of fungal growth conditions using 2% H_2SO_4-pretreated rice straw extract was conducted by varying density, pH, and temperature. Density optimization at 25%, 50%, 75%, and 100% revealed

maximum growth of S1(3) (*Fusarium chlamydosporum* BD-3), S4(9) (*Cladosporium tenuissium ID-4)*, and S6(9) (*Aspergillus aflatoxiformans* IDP-9) at 100% concentration. For pH optimization, S1(3) (*Fusarium chlamydosporum* BD-3) showed maximum biomass at pH 9, S4(9) (*Cladosporium tenuissium ID-4*) at pH 8, and S6(9) (*Aspergillus aflatoxiformans* IDP-9) at pH9. Temperature optimization revealed that incubation at 30°C for seven days in triplicate yielded the highest fungal biomass, confirming that the optimal conditions for fungal growth included 100% rice straw extract, pH 8-9, and 30°C.

The lab-scale and pilot-scale production of fungal bio-cement was carried out using large molds measuring 7 cm × 7 cm, resulting in structurally solid and durable bio-cement bricks. This pilot-scale process demonstrated the contribution of each component: calcium and urea as core reactants and fungi as biocatalysts. The compressive strength of the produced bio-bricks was assessed using a compression testing machine, yielding values of 0 MPa for the control, 15.10 MPa for *Penicillium chrysogenum* (PC), 12.60 MPa for S1(3) (*Fusarium chlamydosporum* BD-3), 10.61 MPa for S4(9) *(Cladosporium tenuissium ID-4)*, and 10.40 MPa for S6(9) (*Aspergillus aflatoxiformans* IDP-9). Water absorption tests revealed absorption rates of 0% for the control, 7.7% for *Penicillium chrysogenum*, 6.9% for S1(3), 5.6% for S4(9) (*Cladosporium tenuissium ID-4*), and 12% for S6(9) (*Aspergillus aflatoxiformans* IDP-9), suggesting that bio-cement bricks produced using these fungal isolates exhibit desirable mechanical and hydrophobic properties.

Advanced instrumental analyses, including ATR-FTIR, XRD, FE-SEM, and TGA spectroscopy, were performed to characterize the chemical, structural, and morphological properties of the bio-cement. ATR-FTIR spectra revealed peaks in the range of 1600–1620 cm^{-1} and 1300–1390 cm^{-1} across all samples (*Penicillium chrysogenum*, S1 (3) (*Fusarium chlamydosporum* BD-3), S4 (9) *(Cladosporium tenuissium ID-4)*, and S6 (9)(*Aspergillus aflatoxiformans* IDP-9), corresponding to the amorphous phases of calcite and aragonite. These peaks were absent in the control, confirming the formation of bio-cement through fungal activity. XRD analysis of the control (sand sample) showed peaks at 2θ values of 20.9°, 26.6°, 36.6°, 42.5°, and 68.2°, corresponding to

crystal planes (100), (011), (110), (200), and (203), indicative of quartz sand. In contrast, fungal bio-cement samples (*Penicillium chrysogenum*, S1 (3) (*Fusarium chlamydosporum* BD-3), S4 (9)(*Cladosporium tenuissium ID-4*), and S6 (9) (*Aspergillus aflatoxiformans* IDP-9) showed additional prominent peaks at 2θ values of 20.917°, 26.8°, and 42.8°, corresponding to crystal planes (100), (011), and (200), confirming the formation of calcium carbonate minerals, specifically calcite. FE-SEM analyses revealed robust microstructures with uniform pore distribution and integration of fungal filaments

within calcium-rich regions, indicating effective biomineralization. SEM images displayed sand particles with rounded, angular, and rectangular geometries. EDS analysis showed that the control sample primarily consisted of silica and oxygen, indicating quartz sand. The EDS spectra of bio-cement samples confirmed calcite formation, with elemental compositions of Ca, C, and O closely resembling that of $CaCO_3$. Thermogravimetric analysis (TGA) further demonstrated the thermal stability of the bio-cement, with total thermal degradation observed as 13.66% for the control, 13% for *Penicillium chrysogenum*, 9% for S1 (3)(*Fusarium chlamydosporum* BD-3), 11% for S4 (9)(*Cladosporium tenuissium ID-4*.), and 8% for S6 (9)(*Aspergillus aflatoxiformans* IDP-9), indicating lower thermal degradation and higher stability in fungal-induced bio-cement.

A wall crack-filling experiment was conducted to evaluate the practical applicability of fungal-induced bio-cement. Over 28 days, S1(3)(*Fusarium chlamydosporum* BD-3)-based bio-cement demonstrated substantial crack reduction, highlighting its potential for construction and restoration applications.

For the molecular identification of the fungal isolates, ITS microbial screening and phylogenetic analysis were performed. The results identified S1 (3) as *Fusarium chlamydosporum*, S4 (9) as *Cladosporium tenuissimum*, and S6 (9) as *Aspergillus aflatoxiformans*. These three fungal species emerged as promising candidates for sustainable bio-cement production and hold potential for further applications in eco-friendly construction practices.

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APPENDIX

RESEARCH PUBLICATIONS AND CONFERENCES

RESEARCH PUBLICATIONS

TITLE: Investigating the potential of delignified rice husk as a carbon rich resource for extracting glucose and its utilization in bio-cement production through fungi isolates

REFERENCE: Devgon, I., Sachan, R. S. K., Kumar, A., Kumar, D., Sharma, A., & Karnwal, A. (2024). Investigating the potential of delignified rice husk as a carbon-rich resource for extracting glucose and its utilization in bio-cement production through fungi isolates. *Environmental Science and Pollution Research*, 1-14.

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ROLE OF CHEMICAL ENGINEERING IN MITIGATION OF ENVIRONMENTAL POLLUTANTS

Investigating the potential of delignified rice husk as a carbon-rich resource for extracting glucose and its utilization in biocement production through fungal isolates

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Abstract
Burning rice straw is now a significant issue faced by different regions in India, as its burning releases harmful gases, mainly carbon dioxide. Various techniques are now in trend to utilize the rice straw, e.g., producing compressed natural gas using rice straw, bioethanol, etc., as a substrate for various microorganisms. A high quantity of non-utilized rice husk generates more ideas for its proper utilization. The cellulose, hemicellulose, and lignin found in rice straws can be a fungi growth medium. In this research, the delignification of rice husk is done by acid (2% and 4% H_2SO_4) and alkali (2% and 4% NaOH) at 121 °C at 103 kPa for 1 h to obtain crude carbon source which is further utilized for biomineralization. The glucose is subjected to qualitative and quantitative analysis using Molisch's and Dinitro salicylic tests. The delignification process showed a positive outcome when 2% H_2SO_4 is utilized maximum yield of 5.9 ug/ml free sugar concentration. Representing the highest glucose yield compared to the experiment's other acid and base substances used. Various techniques such as field emission-scanning electron microscopy (FE-SEM), X-ray diffraction (XRD), and Fourier transformed infra-red (FTIR) spectroscopy are employed to examine surface and chemical alterations. The 2% H_2SO_4 pretreated rice husk is utilized for microbial-induced calcite precipitation using fungal isolates S1 (3), S1 (18), and S4 (1). The calcite and vaterite produced by biomineralization are confirmed using XRD for fungal isolates namely, S1 (3), S1 (18), and S4 (1) having percentage crystallinity of 59%, 46.428%, and 62.69% percentage crystallinity respectively.

Keywords Bioremediation · Fungi · Lignocellulose · MICP · Rice husk · Sustainable

Introduction

The world faces many environmental crises, the burning of fossil fuels for the production of conventional cement emits toxic gases and depletes fossil fuel. Fossil fuel depletion and the negative impact of non-renewable energy sources on the environment have led to an urgent need for alternative renewable energy. Any renewable resource that can produce bioenergy with low greenhouse gas emissions is crucial to addressing today's environmental issues. Lignocellulose biomass has been identified as a promising renewable source of bioenergy due to its ability to recover nutrients at a higher rate than it emits greenhouse gases (Anil et al. 2020). Lignocellulose biomass refers to plant-based materials that contain lignin, cellulose, and hemicellulose. These materials are widely available, making them a cost-effective and accessible alternative to fossil fuels.

However, using lignocellulosic biomass for bioenergy production necessitates converting its resistant structure into fermentable sugars. To enhance the sugar yield, scientists have proposed several pretreatment methods. These approaches aim to dismantle the recalcitrant connections

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Exploring fungal potential for microbial-induced calcite precipitation (MICP) in bio-cement production

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Introduction: Microbial-induced calcite precipitation (MICP) involves various microorganisms, such as bacteria, fungi, and algae. This study focuses on producing bio-cement using fungal species and selecting potential candidates isolated from alkaline soil of different regions of Punjab, namely, Majha, Malwa, and Doaba.

Methods: The selection of fungi isolates capable of bio-cement production involves several tests, including a urease assay and calcium precipitation. Isolates having high urease enzyme production and the ability to perform calcite precipitation are selected for instrumental analyses such as X-ray diffraction (XRD) and scanning electron microscopy (SEM). The isolates selected for further analysis are S1 (3) with 8.879 ± 2.94 µg/ml, S1 (18) with 8.421 ± 0.13 µg/ml, and S4 (1) with 10.057 ± 0.45 µg/ml urease activity and least free calcium ions that are 2.337 ± 0.5 µg/ml, 3.339 ± 0.5 µg/ml, and 4.074 ± 0.1 µg/ml respectively.

Results and discussion: Calcite precipitation is confirmed through XRD and field emission scanning electron microscopy (FESEM). XRD images showing calcite precipitation with sharp crystalline peaks for S1 (3), S1 (18), and S4 (1) are shown. The calcite precipitation is evident in the micrographs of FESEM. These combined results confirm the potential of urease-positive fungi to facilitate calcite production, which could lead to bio-cement development in future research.

KEYWORDS

fungal calcite precipitation, urease-positive fungi, bio-cement production, environmental mineralization, fungal bio-mineralization

Highlights

- Exploring fungal-driven MICP for bio-cement production.
- Sample collection from Majha, Malwa, and Doaba regions.
- Selection of fungi through urease assay and calcium precipitation tests.

CONFERENCES

1. Participation in Oral Presentation on ‘Selection of potential fungus for bio-concrete production: a sustainable approach” in International Conference on “Microbial Bioprospecting Towards Sustainable Development Goals” held at Lovely Professional University (2023).
2. Participation in Oral presentation on “Characterization of alkali-based and acid-based treatment of rice straw” in International Conference on “Bioengineering and Biosciences (ICBB)” held at Lovely Professional University (2023).

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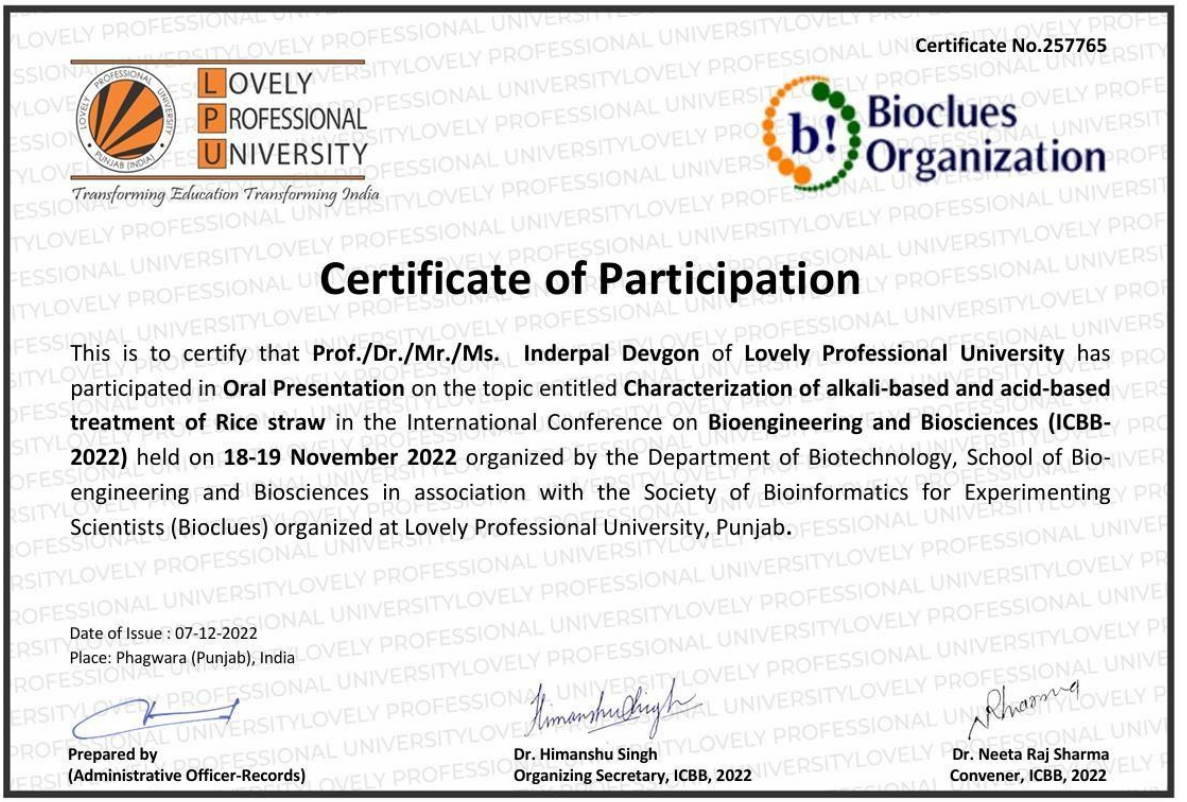

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