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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.

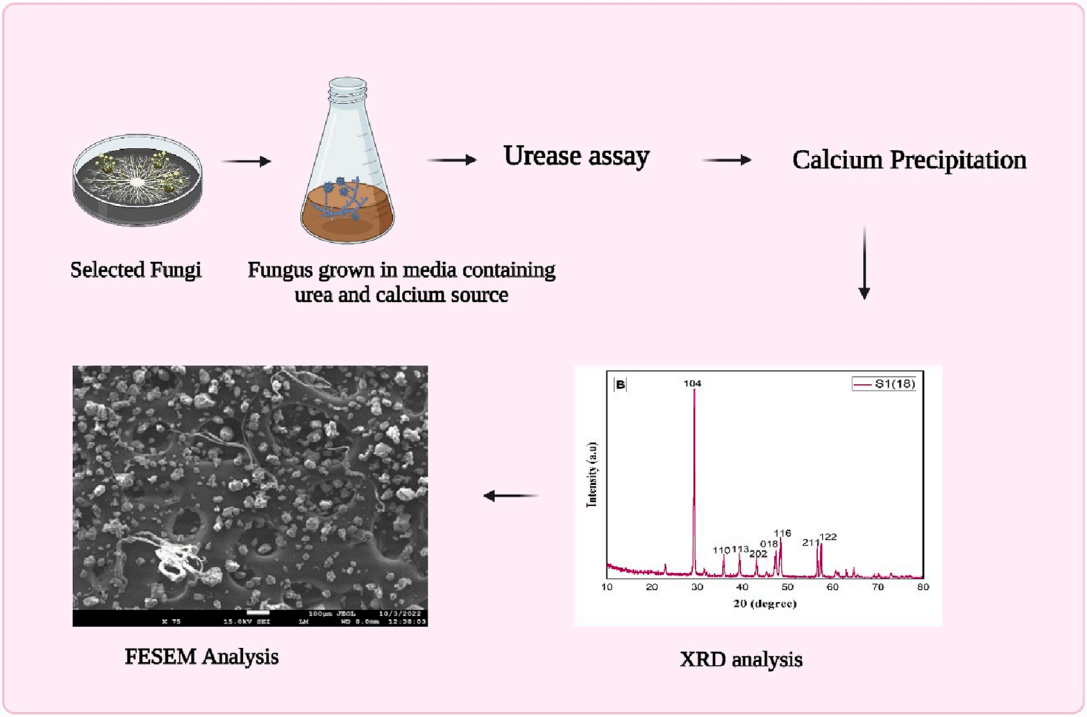
Results and discussion: Calcite precipitation is confirmed through XRD and field emission scanning electron microscopy (FESEM). XRD images showing calcite precipitation with sharp crystalline peaks for S1 (3), S1 (18), and S4 (1) are shown. The calcite precipitation is evident in the micrographs of FESEM. 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.



GRAPHICAL ABSTRACT

- Isolates S1 (3), S1 (18), and S4 (1) showed high urease activity and low free calcium ions.
- X-ray diffraction (XRD) and scanning electron microscopy (SEM) confirmed calcite precipitation.

1 Introduction

Microorganisms have been utilized in a variety of industries, including food, medicine, and cosmetics (Rajput et al., 2022). The global construction industry is looking for eco-friendly alternatives to traditional cement production due to concerns over its high carbon footprint and impact on greenhouse gas emissions (Huntzinger and Eatmon, 2009). Cement manufacture requires high temperatures, usually between 1,400°C–1,500°C, which leads to the combustion of fossil fuels and the release of hazardous gases such as sulfur dioxide, carbon dioxide, and carbon monoxide (Tosti, 2021). The industrial process of cement production from lime, which is a precursor of concrete, consumes 2%–3% of the world's energy demand. This process generates 0.73–0.99 tons of CO_2 per ton of cement produced, contributing to approximately 8%–10% of global anthropogenic CO_2 emissions and 3.4% of total global CO_2 emissions (dos Santos et al., 2021; Zhao et al., 2022). The rise in concrete consumption is due to infrastructures being vulnerable to various physical, chemical, and biological factors like temperature changes, exposure to corrosive and radioactive materials, aggressive gases, natural calamities, and microbial processes (Ye et al., 2023; Lin et al., 2023; Zhang et al., 2024). Conventional cement is susceptible to early-age cracking from environmental conditions, a problem that can be reduced by using bio-cement, which has self-healing capabilities (Safiuddin et al., 2018; Zhang et al., 2024). The rise of bio-cement manufacturing using urease-positive fungi offers a hopeful and sustainable alternative in this context.

Microbially induced carbonate precipitation (MICP) is a natural biochemical process where microorganisms trigger the creation of calcium carbonate precipitation. Microorganisms initiate carbonate precipitation by utilizing different metabolic processes such as photosynthesis, ureolysis, ammonification, denitrification, sulfate reduction, anaerobic sulfide oxidation, and methane oxidation (Castro-Alonso et al., 2019). The routes include mechanisms that elevate pH levels and enhance the concentration of dissolved inorganic carbon (DIC) (Martuscelli et al., 2020; Devgon et al., 2024). Bio-cementation procedures utilize microbially induced calcium carbonate precipitation (MICP) to solidify sand by generating calcium carbonate, leading to varied desired consequences. Calcium carbonate precipitates to create the bonding substance that holds particles together. The primary metabolic pathway of interest is urea hydrolysis, which is commonly utilized in significant research investigations (Tosti, 2021). The process consists of three clear stages: (i) introducing microbes into the medium, (ii) injecting a cementation solution with urea and a calcium source, and (iii) causing the cementing agent to precipitate, binding the sand particles and enhancing strength and stiffness (Konstantinou and Wang, 2023).

The research gap in the literature is highlighted by the limited exploration of fungal species for microbial-induced

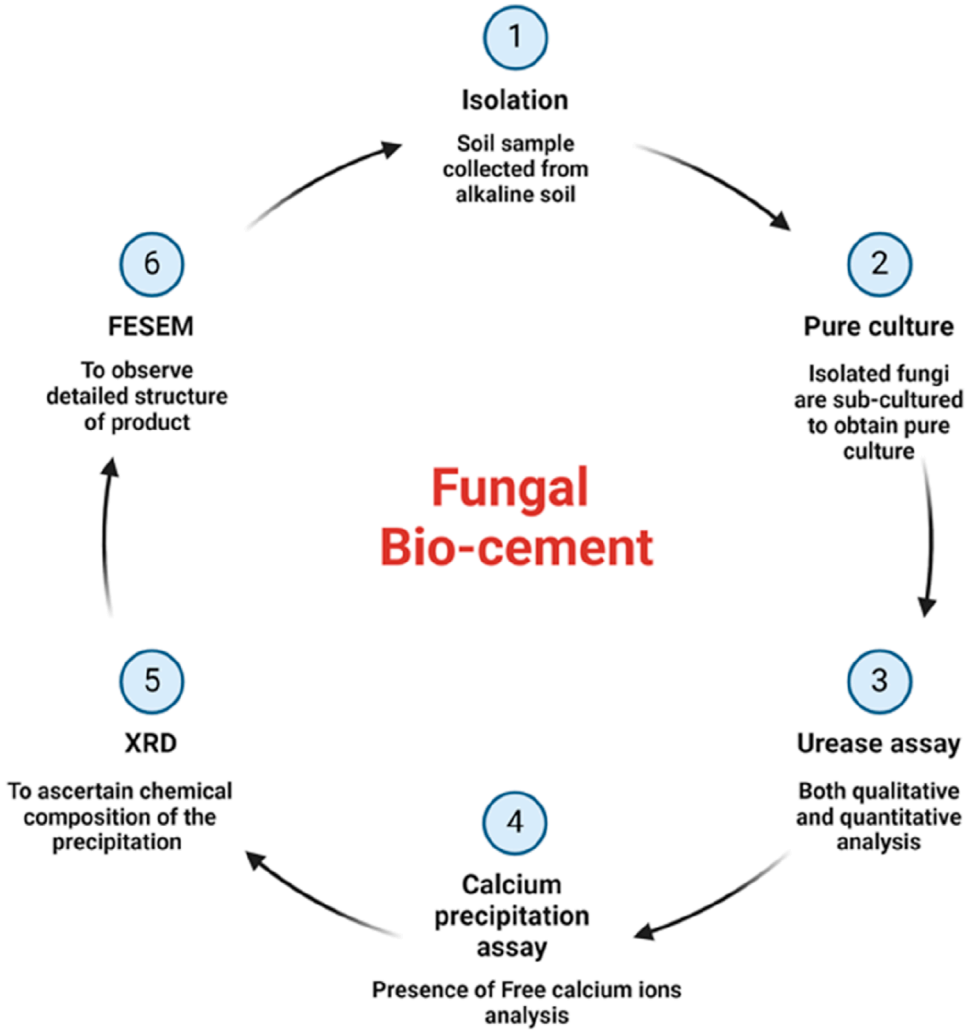


FIGURE 1
Schematic diagram of the isolation and production of bio-cement using fungi.

calcite precipitation (MICP) compared to the extensive focus on bacteria. Additionally, there is a lack of detailed understanding of fungal mechanisms for calcite production and their practical application in bio-cement. This work fills these gaps by investigating fungal candidates and providing new insights into their potential for sustainable bio-cement development.

TABLE 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 L

Urease-positive fungi can generate urease enzymes, which are essential for converting urea into carbonate ions and ammonia (Fang et al., 2018; Konstantinou and Wang, 2023; Li et al., 2023). This enzymatic activity can be utilized to stimulate mineral precipitation, specifically the creation of calcium carbonate, an essential element in bio-cement manufacturing (Iqbal et al., 2021). Utilizing urease-positive fungi for bio-cement addresses environmental issues of traditional methods and provides a sustainable and eco-friendly alternative for construction while also tackling early-age cracking problems in regular cement (Lin et al., 2023).

This study pioneers using fungal species, rather than bacteria, for MICP in bio-cement production. Exploring

TABLE 2 Media composition used for calcium precipitation assay.

Sr No.	Chemical	Concentration	Manufacturer	Gram/L
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 sulfate	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 sulfate	-`	Loba Chemie Pvt. Ltd	4 mg
8	Manganese sulfate	1.8 x 10–^{2} mM	Loba Chemie Pvt. Ltd	4 mg
9	Copper sulfate	1.6 x 10–^{3} mM	Loba Chemie Pvt. Ltd	0.4 mg
10	2% H_2SO_4-treated rice husk extract	—	—	100 mL

TABLE 3 Urease enzyme produced in µg/mL by different urease-positive fungi.

S. No.	Isolate	Concentration (µg/mL)
1	S5 (9)	2.204188 ± 0.85
2	S5 (6)	2.858639 ± 0.19
3	S6 (1)	2.858639 ± 1.11
4	S6 (14)	2.924084 ± 0.39
5	S2 (7)	3.316754 ± 0.39
6	S2 (4)	3.513089 ± 0.32
7	Pc	4.429319 ± 0.06
8	S5 (3)	4.691099 ± 0.98
9	S5 (10)	4.887435 ± 0.65
10	S4 (3)	4.82199 ± 0.45
11	S6 (7)	4.82199 ± 0.45
12	S5 (1)	5.21466 ± 0.32
13	S6 (16)	6.65445 ± 0.58
14	S6 (8)	7.505236 ± 0.26
15	S1 (28)	7.832461 ± 1.76
16	S4 (9)	7.832461 ± 1.11
17	F	8.290576 ± 0.65
18	S6 (9)	9.926702 ± 0.98
19	S1(18)	8.421466 ± 0.13
20	S1(3)	8.879581 ± 2.94
21	S4(1)	10.05759 ± 0.45

TABLE 4 Free calcium ion concentrations after inoculation of urease-positive fungi and incubation at 30°C for 7 days. When fewer free calcium ions are present, more calcite precipitation has occurred.

Sr No.	Sample name	Free calcium ions mM/50 mL
1	S1 (3)	2.337883 ± 0.5
2	S1 (18)	3.339833 ± 0.5
3	S4 (1)	4.074597 ± 0.1
4	S1 (28)	4.341783 ± 0.1
5	S2 (7)	6.345683 ± 0.5
6	S2 (8)	10.35348 ± 0.5
7	S2 (4)	11.35543 ± 2.3
8	S1 (1)	22.37688 ± 0.5
9	S3 (7)	22.0429 ± 2.0
10	S3 (5)	24.0468 ± 2.0
11	S4 (3)	37.40613 ± 3.0

urease-positive fungi and their involvement in bio-cement is crucial for comprehending their significant influence on construction methods and environmental preservation when selecting candidates for microbial-induced calcite precipitation. Isolating fungi from alkaline soils and confirming their calcite precipitation through XRD and field emission scanning electron microscopy (FESEM) expands the scope of microorganisms in bio-cement research (Figure 1). The research highlights fungi as promising candidates for sustainable bio-cement production, offering an alternative to bacteria with potential benefits like enhanced environmental tolerance. This opens new avenues for eco-friendly construction materials and future biotechnological applications.

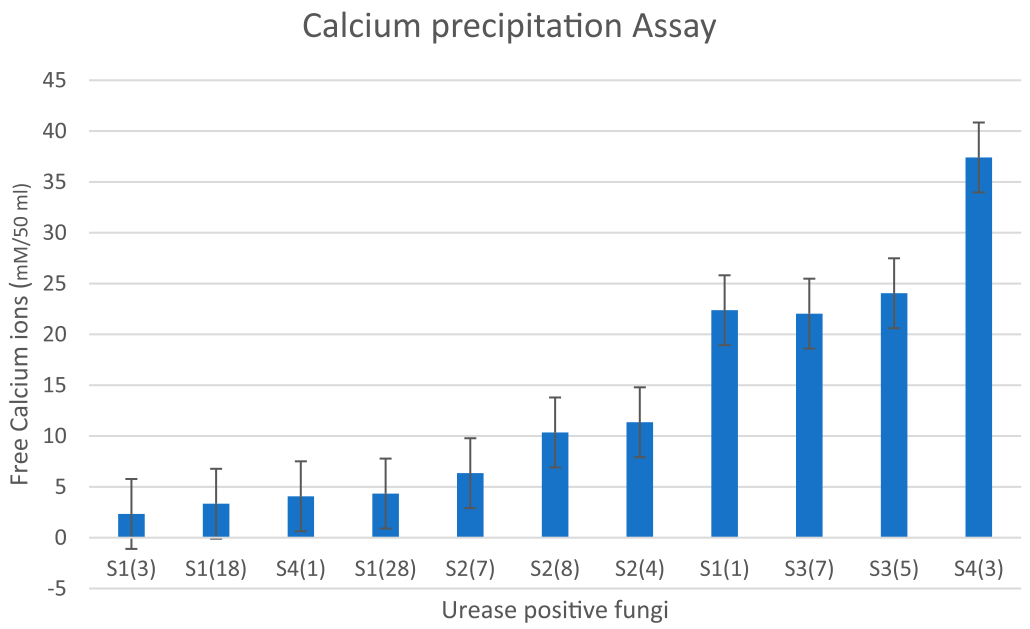


FIGURE 2
Utilization of free calcium ions by fungal samples.

2 Materials and methods

2.1 Isolation

The alkaline soil samples are collected from specific locations in the Punjab region: Phagwara (latitude 31.249168°, longitude 75.709499°), Moga (latitude 30.966159°, longitude 75.481656°), Muktsar (latitude 30.49625°, longitude 74.571997°) and Amritsar (latitude 31.499645, longitude 75.321991°). The soil samples are collected in a sterile polybag and stored at 4°C (Chaudhary et al., 2013). For isolation, 1 g of soil is inoculated in 10 mL autoclaved broth for 24 h at 27°C. Afterward, serial dilution is performed, and 100 µL of sample is used from both 10^{-5} and 10^{-6} tubes to spread aseptically on prepared potato dextrose agar plates containing chloramphenicol (antibacterial agent) to avoid bacterial contamination. Hyphae tips freshly grown in potato dextrose agar (PDA) plates are used to obtain pure isolates on freshly prepared autoclaved PDA plates (Acharya and Hare, 2022). All pure culture slants are prepared in slant tubes using PDA media and are stored at 4°C, and glycerol stocks are stored at −80°C (Kitamoto et al., 2002).

2.2 Urease assay

2.2.1 Qualitative urease test

A method is employed to assess the fungi's potential to induce calcite precipitation through urease enzyme activity. Autoclaved Christensen's agar medium and urea broths are prepared to isolate urease-positive strains. Urease activity is observed by observing a distinct color shift (FAEZI et al., 2004), specifically from yellow to pink, which serves as a positive indication. This procedural method is utilized to selectively identify fungal isolates proficient in urease enzyme production, paving the way for subsequent investigations into their calcite precipitation capabilities (Zhao et al., 2022).

2.2.2 Quantitative urease assay

A quantitative urease assay is performed to calculate the amount of urease enzyme produced in µg/mL by different isolates. The chemicals used for the assay are given below in Table 1.

Following the preparation of autoclaved media, isolates are inoculated using a cork borer for a 7-day incubation period at 30°C. After this duration, mycelia are homogenized with a homogenizer and filtered through Whatman filter paper. The mycelia obtained on the filter paper are washed using 1 M potassium phosphate buffer, followed by a wash with 0.1 M 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). A 50 µL aliquot of the reaction mixture is 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°C. Absorbance at 630 nm is measured, and the values are calculated using a standard ammonium sulfate graph. This detailed procedure ensures the accurate quantification of urease activity in the analyzed samples.

2.3 Calcium precipitation assay

Examining calcium precipitation entails titration with EDTA, Eriochrome black T dye, sodium hydroxide buffer, and HCL.

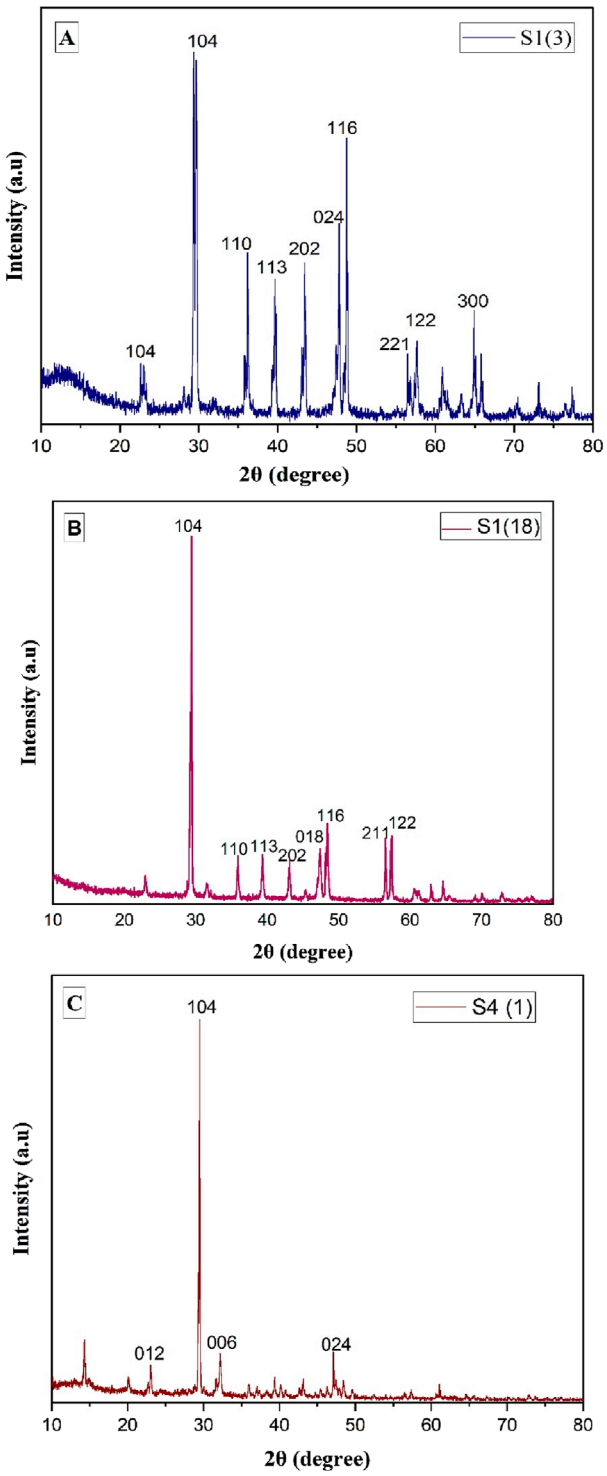


FIGURE 3
XRD graph of calcium precipitation for isolates **(A)** S1 (3), **(B)** S4 (1), and **(C)** S18. The peak corresponding to 104 indicates calcium carbonate.

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 blue, indicating the completion of the calcium ion reaction with EDTA. This titration is done in triplicate for precision, and the average volume of EDTA used is 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 following formula:

Calculation of free calcium ions is calculated using the following formula:

1 mL EDTA × molarity EDTA = mmoles EDTA = mmoles Ca_2^+ × 40.078 g/mol = mmoles Ca_2^+,

aliquot 2(Ca^{2+} mg, aliquot) × (50.00 mL 250.00 mL) = Ca^{2+} mg, unknown.

The growth media to perform the calcium precipitation is given below in Table 2.

2.4 Instrumentation analysis

2.4.1 X-ray diffraction (XRD)

XRD is used to ascertain the chemical composition of the precipitation resulting from bacterial mineralization, as detailed by Fang et al. (2018). The crystal structure of the bio-cement formed by microorganisms is elucidated through XRD, following the methodology outlined by Anitha et al. (2018). To investigate the presence of calcium carbonate on the hyphae of ureolytic fungi, a diffractometer equipped with a Bruker D8 Advance is employed for the structural analysis of the fungus hyphae. The analysis is performed after a 7-day incubation period to observe any discernible calcium carbonate presence on the hyphae.

2.4.2 Field emission scanning electron microscopy (FESEM)

Field emission scanning electron microscopy (FESEM) is employed to examine the bio-cement generated by fungal isolates, as described by Fang et al. (2018). The visualization using FESEM allows for a detailed observation of the structure.

3 Results and discussion

3.1 Isolation

In microbial-induced calcite precipitation, the selected fungal strain must be urease-positive, as this research focuses on the production of calcite through urea hydrolysis. Of the different fungi isolated from the soil sample, 21 are urease-positive (Li et al., 2015).

3.2 Screening potential candidates based on urease assay and calcium precipitation

3.2.1 Qualitative urease assay

After inoculation in Christensen's medium, a color change is observed to select urease-positive fungi. The color shifts from yellow to pink due to the hydrolysis of urea by the enzyme urease, which is produced by the fungus. Urease catalyzes the breakdown of urea into ammonia and carbon dioxide. The

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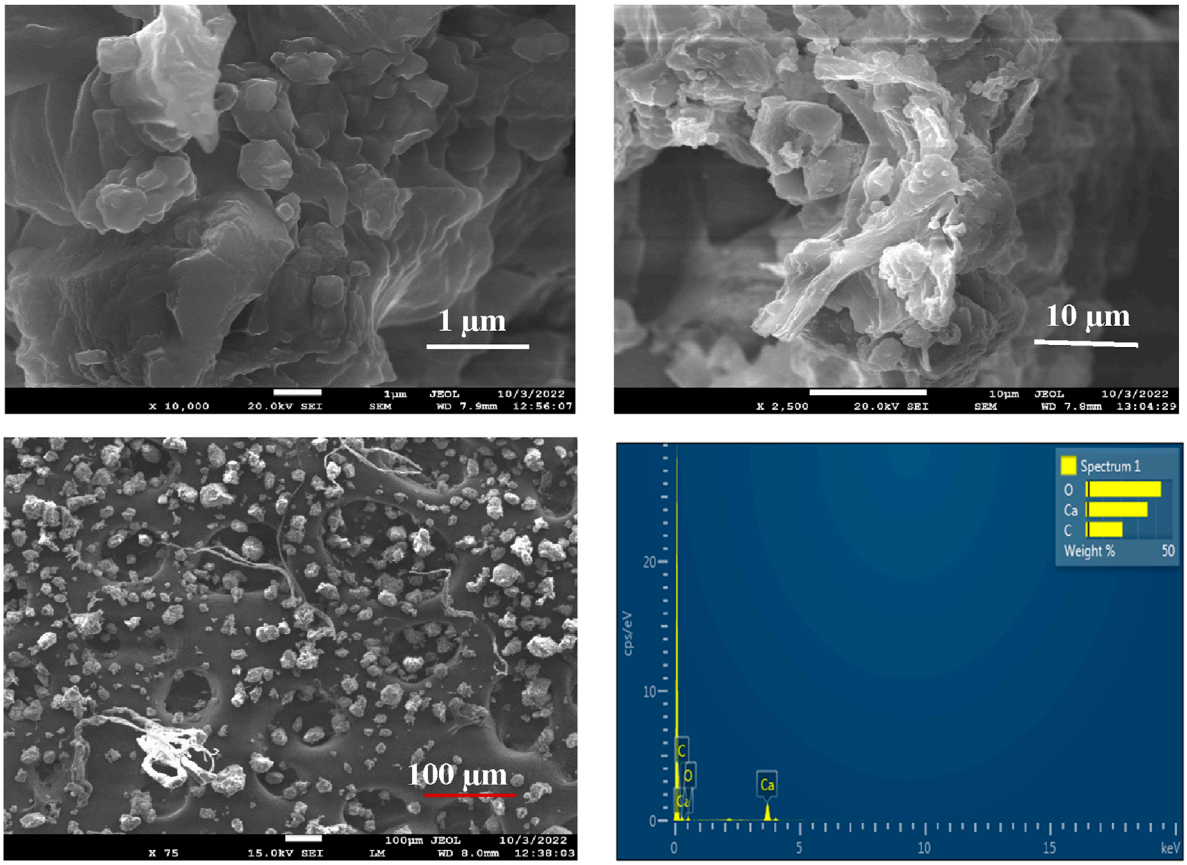
TABLE 5 Total crystalline index of selected fungi.

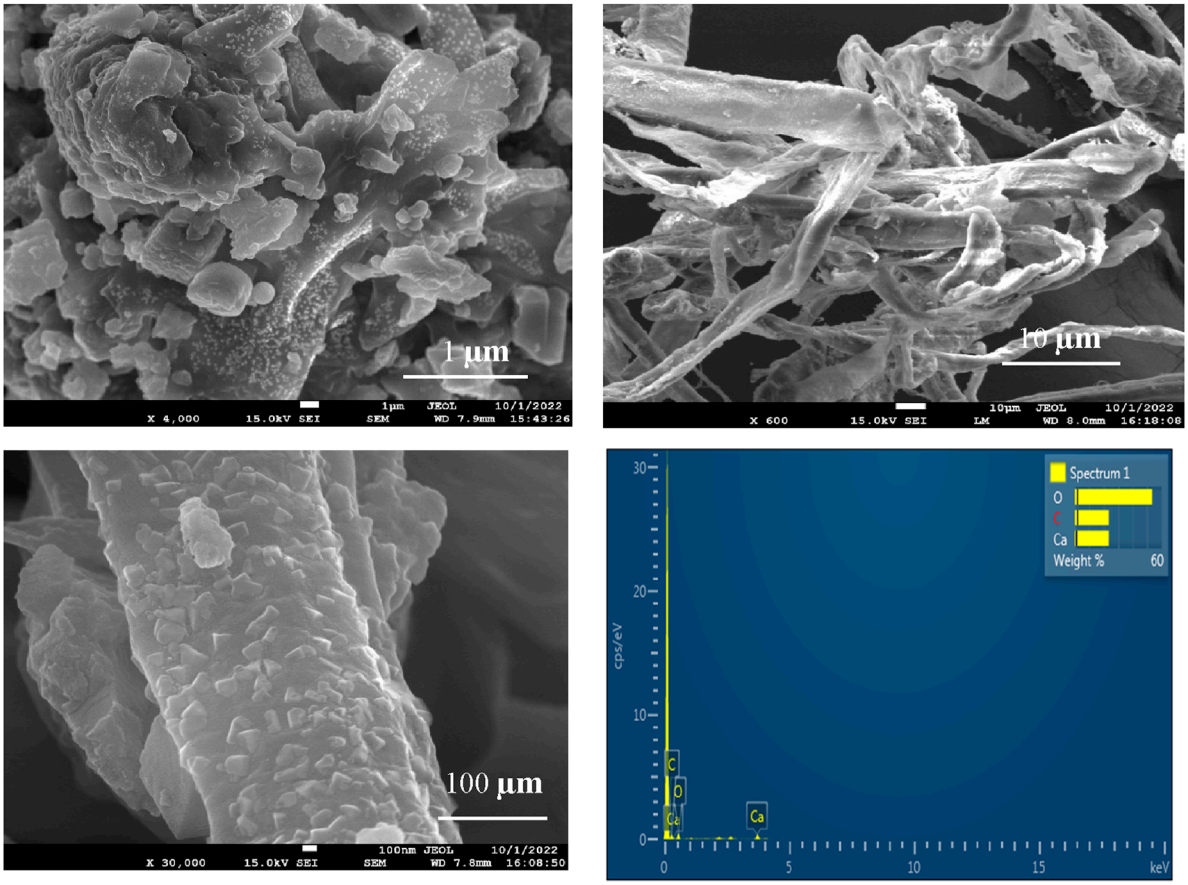
S.No	Name	Crystalline area	Amorphous area	X% (crystalline)
1	S1 (3)	1,461.44	1,015.57	59
2	S1 (18)	1714.96	1978.04	46.4
3	S4 (1)	1888.84	1,123.77	62.6



Spectrum 1	Wt.%	Wt.% Sigma
C	21.08	1.62
O	43.28	1.79
Ca	35.64	1.29

Spectrum	Atomic %
C	32.80
O	50.57
Ca	16.62
Total	100.00

FIGURE 4
SEM and EDS spectra of calcite precipitation in fungal isolate S1 (3), showing deposited crystals.



Spectrum 1	Wt.%	Wt.% Sigma
C	23.36	1.97
O	53.31	1.89
Ca	23.33	1.16

Spectrum	Atomic %
C	33.20
O	56.87
Ca	9.93
Total	100.00

FIGURE 5
SEM and EDS spectra of calcite precipitation in fungal isolate S (18), showing deposited crystals.

ammonia produced increases the pH of the medium, shifting it from an acidic to an alkaline environment. The urea broth contains the pH indicator phenol red, which is yellow under acidic conditions and turns pink in alkaline conditions, indicating a positive result (Martuscelli et al., 2020; Zhang et al., 2024). Of all the isolates, 21 are urease-positive, and a quantitative urease assay was performed to identify the highest producers of the urease enzyme.

3.2.2 Quantitative urease assay

After inoculation of isolates for 7 days at 30°C using a standard ammonia graph, concentrations in µg/mL were calculated

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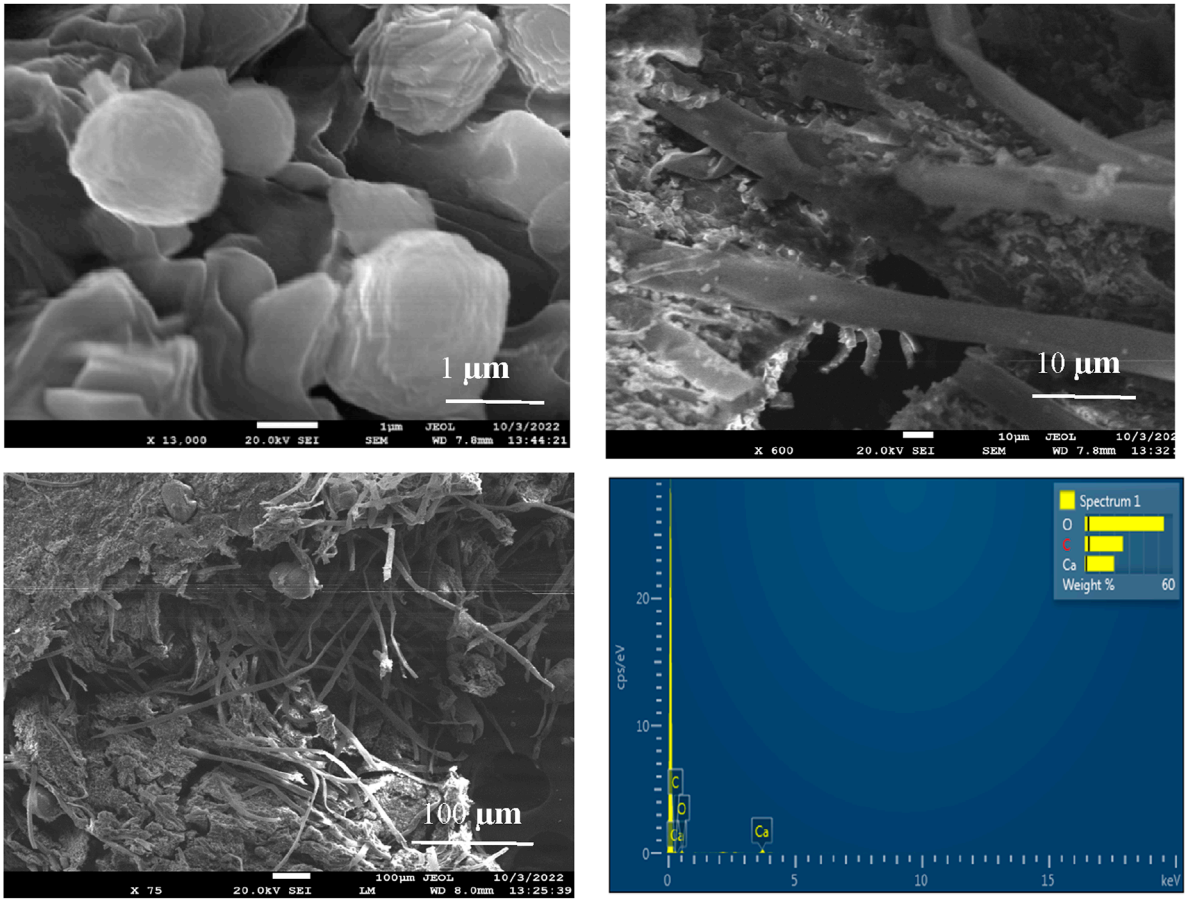

Spectrum 1	Wt.%	Wt.% Sigma
C	26.17	2.98
O	53.78	2.80
Ca	20.05	1.35

Spectrum	Atomic %
C	36.07
O	55.65
Ca	8.28
Total	100.00

FIGURE 6
SEM and EDS spectra of calcite precipitation in fungal isolate S4 (1), showing deposited crystals.

as shown in Table 3 for all the isolates. An ammonia standard graph, or standard curve, is used to determine the amount of urease activity in a sample by plotting the values from ammonium chloride standards. It is observed that S1 (3), S1 (18), S4 (1), S4 (9), S6 (8), S6 (9), and F are the maximum producers of urease enzyme.

3.3 Calcium precipitation/cementation assay

The fungal isolate must be able to precipitate calcium for the MICP process. High urease enzyme producers will precipitate

more calcium. After performing the urease assay, the high urease producers are further inoculated in growth media containing a calcium source and urea and incubated for 7 days at 28°C. It is observed that S1 (3), S1 (18), S4 (1), S1 (28), S2 (7), and S2 (8) showed maximum removal of free calcium ions in the solution with the lowest number of free calcium ions as shown in Table 4 (Figure 2). Based on the results of both the urease assay and the calcium precipitation assay, S1 (3), S1 (18), and S4 (1) are identified as isolates having higher urease enzyme production that can precipitate calcium and are selected for instrumentational analysis (Akoijam et al., 2021).

3.4 Instrumentation analysis

3.4.1 XRD analysis of calcite precipitation

The distinct peaks of the graphical representation of various fungal isolates strongly indicate the ability of S1 (3), S1 (18), and S4 (1) f to promote calcite precipitation. Specifically, the sharp peak near 29.3° for 2θ provides valuable insights into the crystallinity of calcite (calcium carbonate).

Specific 2θ values observed for S13 (29.3, 39.95, 39.3, 43.1, 47, 48, 56.5, 57.3), S41 (29.3, 31.4, 23, 47), and S1 (18) (29, 35.9, 39, 43.1, 47, 48.4, 56, 57.3) shown in Figure 3. These findings underscore the role of these fungal isolates in promoting the precipitation of calcium carbonate on their hyphae. Crystalline size is calculated and shown in Table 5.

3.4.2 SEM analysis of calcite precipitation

Figures 3–6 depict the SEM analysis of calcite precipitation, revealing the presence of larger mineral crystals within fungal isolates S1 (13), S1 (18), and S4 (1). These crystals serve as tangible evidence of the active involvement of fungal isolates in the formation of calcite precipitation (Burford et al., 2006). The localized accumulation of calcite near fungal hyphae suggests the existence of nucleation sites for mineralization (Fomina et al., 2006; Ye et al., 2023). Further confirmation of calcite presence was obtained through energy dispersive spectroscopy (EDS), which identified the elements Ca, C, and O, closely resembling the atomic composition of $CaCO_3$.

4 Conclusion

This work emphasizes the promising ability of fungi to produce cement through microbially induced calcite precipitation (MICP). Although the traditional focus of MICP research has been on bacteria, our study emphasizes the unique advantages of fungi. They are promising candidates for boosting calcite precipitation owing to their abundance of nucleation sites and ability to survive for a prolonged time as spores. We isolated urease-positive fungi from different areas of Punjab, including Majha, Malwa, and Doaba. We applied a systematic selection process to discover key isolates, S1 (3), S1 (18), and S4 (1), that revealed noteworthy calcite precipitation capabilities. Comprehensive testing, including assays for calcium precipitate and instrumental evaluations with XRD and scanning electron microscopy (SEM), are used to confirm the evidence of calcite precipitation. Notably, isolates S1 (3), S1 (18), and S4 (1) displayed significant calcium precipitation. Conclusively, these outcomes highlight that the selected urease-positive fungi can produce calcite precipitation.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material; further inquiries can be directed to the corresponding authors.

Author contributions

ID: Formal Analysis, Investigation, Methodology, Resources, Visualization, Writing–original draft. RK: Data curation, Formal Analysis, Methodology, Software, Validation, Writing–original draft. KR.: Data curation, Formal Analysis, Investigation, Validation, Writing–original draft. MK: Data curation, Methodology, Resources, Writing–original draft. AM: Data curation, Formal Analysis, Validation, Writing–original draft. AK: Conceptualization, Formal Analysis, Resources, Supervision, Writing–original draft, Writing–review & editing. TM: Data curation, Formal Analysis, Resources, Visualization, Writing–review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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