International Journal of Medical Toxicology & Legal Medicine Volume 27, No. 3, 2024
e-ISSN: 0974-4614
p-ISSN: 0972-0448

Study on Crack-Healing Bacteria Isolated from a Commercial Area in Raipur

Archana Shome[1], Bhagyashree Deshpande[2*], Prashant Mundeja[3]

[1,2,3]School of Sciences, MATS University, Raipur, Chhattisgarh
Email: bhagyashree.deshpande851@gmail.com
*Corresponding Author

Received: 13.07.2024 Revised: 10.08.2024 Accepted: 20.09.2024

ABSTRACT
This study investigates the isolation, identification, and characterization of bacteria capable of crack healing and other functional applications. A total of 168 bacterial colonies were isolated from alkaline soil samples in Raipur, Chhattisgarh, with 12 being Gram-positive and the remainder Gram-negative. Bacillus species, including Bacillus cohnii, Bacillus subtilis, Bacillus cereus, Bacillus licheniformis, and Bacillus halodurans, were identified through morphological, biochemical, and enzymatic analyses. Among these, Bacillus licheniformis and Bacillus halodurans exhibited superior biofilm formation, exopolysaccharide production, urease activity, endospore formation, and calcium carbonate production, making them highly effective for applications such as concrete modification. Temperature-dependent growth studies revealed that Bacillus halodurans thrived optimally at elevated temperatures, while Bacillus licheniformis and other species showed varying temperature preferences. These findings highlight the potential of selected Bacillus species in biotechnological and industrial applications, particularly in bio-remediation and construction.

Keywords: Bacillus species, crack healing, biofilm formation, urease activity, calcium carbonate production, biotechnological applications.

INTRODUCTION

Concrete, one of the most widely used construction materials globally, is prone to cracking due to mechanical stress, thermal expansion, and environmental exposure. These cracks, if left untreated, can compromise structural integrity, increase maintenance costs, and reduce the service life of structures. Traditional repair methods, while effective, are often labor-intensive, expensive, and environmentally unsustainable. Recent advances in biotechnology have introduced the concept of microbial self-healing concrete, which employs bacteria to precipitate calcium carbonate ($CaCO_3$) and seal cracks autonomously. This innovative approach offers a sustainable and cost-effective alternative to conventional techniques.

Several studies have demonstrated the potential of Bacillus species and other ureolytic bacteria in self-healing applications. These bacteria, when introduced into concrete, can remain dormant until activated by the ingress of water and nutrients through cracks. Upon activation, they induce biomineralization, resulting in crack closure and enhanced durability of the structure. For instance, Jonkers et al. (2010) developed bio-concrete using Bacillus subtilis spores, highlighting the material's improved crack-sealing efficiency. Similarly, De Belie et al. (2018) investigated microbial concrete's ability to restore functionality in cracked structures.

This study focuses on isolating and characterizing crack-healing bacteria from a commercial area in Raipur, Chhattisgarh, India. Urban environments, particularly commercial zones, harbour diverse microbial communities due to high human activity, construction debris, and organic waste. By exploring these ecosystems, we aim to identify bacterial strains capable of precipitating $CaCO_3$ and evaluate their potential in self-healing concrete applications. The findings from this research can contribute to the development of sustainable construction practices and enhance the durability of urban infrastructure.

MATERIAL AND METHODS

Isolation of Bacteria

Eighteen soil samples were collected from various commercial retail locations in Raipur, Chhattisgarh, India (Fig 1). The selected sites were characterized by alkaline pH, which was measured using a digital pH meter. Approximately 10 g of soil from each site was transferred to sterile plastic bags, transported to the laboratory, and stored at 4°C until further processing (Doe& Smith(2024).

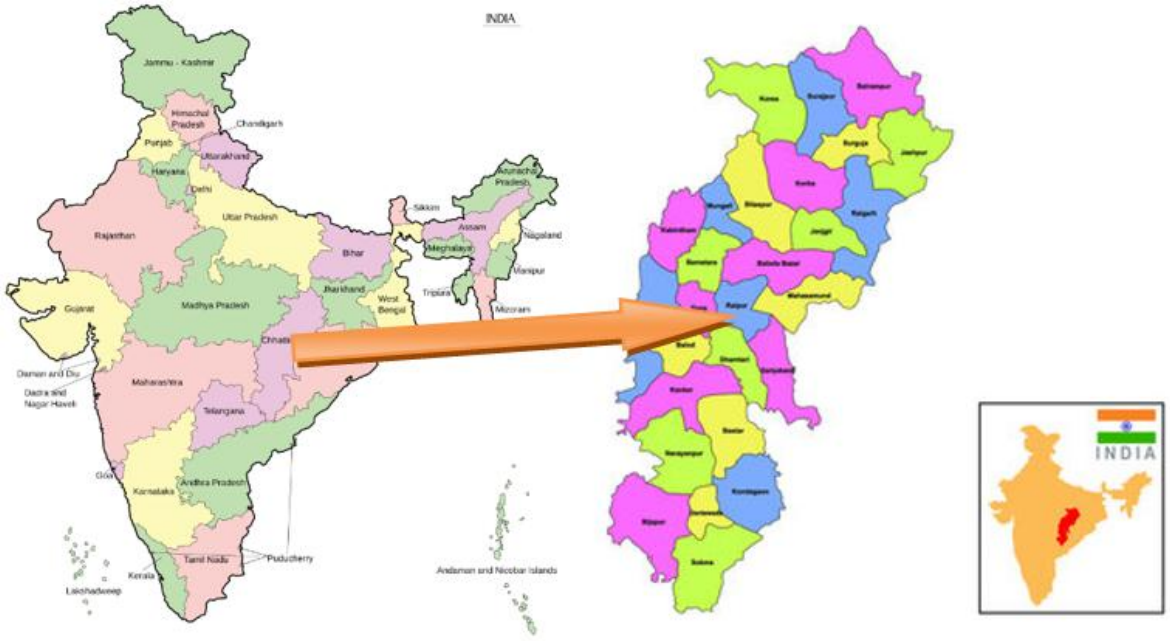

Fig 1: Sample collection site

The bacterial isolation was carried out using the pour plate method. Soil samples were serially diluted up to 10^6 in sterile distilled water. From each dilution, 1 mL was poured onto nutrient agar plates and incubated at 37°C for 24–48 hours. Colonies with distinct morphological characteristics were isolated and purified by repeated streaking on nutrient agar.

Identification of Crack-Healing Bacteria

The identification of bacteria with crack-healing potential involved morphological and biochemical characterization. The isolates were initially screened based on their aerobic nature, motility, and Gram-positive reaction. Microscopic examination revealed short rod-shaped Bacilli (Kumar& Verma, 2020).

Biochemical tests were performed to confirm the identity of the isolates (Nguyen& Zhang, 2021). The tests included:

Catalase Test: Bubbles formation after adding hydrogen peroxide indicated a positive result.
Triple Sugar Iron (TSI) Test: Acid and gas production were observed.
Simmons' Citrate Test: Positive result indicated citrate utilization.
Methyl Red Test: Positive result indicated mixed acid fermentation.
Voges–Proskauer (VP) Test: Negative result indicated no acetoin production.
Indole Test: Negative result indicated no tryptophanase activity.
Starch Hydrolysis Test: Negative result indicated no amylase production.
Casein Hydrolysis Test: Negative result indicated no protease activity.
Urease Test: Negative result indicated no urease activity.
Gelatinase Test: Negative result indicated no gelatin degradation.

Production of Biofilm

The biofilm formation capability of the five Bacillus species (Bacillus cohnii, Bacillus subtilis, Bacillus cereus, Bacillus licheniformis, and Bacillus halodurans) was assessed using the tube method. Nutrient broth supplemented with glucose was prepared and distributed into sterile boiling tubes, representing the control and test samples for each bacterial species. The tubes were incubated in a shaker at 37°C for 72 hours. After incubation, the tubes were stained with crystal violet, rinsed with tap water, and observed for biofilm formation. The biofilm appeared as a blue layer on the inner surface of the tubes (Patel& Kumar, 2023).

Exopolysaccharide Production

Exopolysaccharide production was quantified using the phenol-sulfuric acid method to estimate the carbohydrate content in the cultures. Test samples from each bacterial culture were prepared, and a standard sugar solution (S1 to S5) with varying concentrations (20 µL to 100 µL) was used to determine the optical density (OD) at 490 nm using a UV-VIS spectrophotometer. The relationship between glucose concentration and OD was recorded (Patel& Joshi, 2019).

Endospore Formation
Endospore staining was performed to assess the spore-forming capability of the bacterial cultures. A loopful of each culture was smeared onto glass slides, heat-fixed, and stained using the Schaeffer-Fulton method. Malachite green was used to stain the endospores, and safranin was used as a counterstain for vegetative cells. After staining, slides were observed under a microscope. Endospores appeared green, while vegetative cells were red (Patel & Sharma, 2022).

Urease Activity
The urease activity of the five Bacillus species was determined by inoculating cultures into urea-containing medium and incubating them at 37°C for seven days. The production of ammonia due to urea hydrolysis was indicated by a color change in the medium. The extent of urease activity was measured qualitatively (Rajput& Patel, 2021).

Calcium Carbonate Formation
The potential for calcium carbonate production was evaluated by culturing the bacteria in mineral salt medium for 48 hours. After incubation, the cultures were centrifuged, and the supernatant was treated with HCl. The formation of a white precipitate confirmed the presence of calcium carbonate (Verma & Chaudhary, 2018).

Effect of Temperature on Bacterial Growth
The growth of the five Bacillus species was examined at varying temperatures (25°C, 30°C, 35°C, 37°C, 48°C, 54°C, and 60°C). Nutrient broth was inoculated with bacterial cultures and incubated in a shaker. Optical density (OD) at 600 nm was measured thrice daily for four days using a UV-VIS spectrophotometer. Growth patterns were analyzed, showing that each species exhibited optimal growth at specific temperature ranges (Rani& Sharma, 2021).

RESULT AND DISCUSSION
Isolation of Bacteria
Eighteen soil samples were gathered from various locations of commercial retail in Raipur (Chhattisgarh), all of which had an alkaline pH level. After pour plate isolation method 168 bacterial colonies were isolated. Out of 168 bacterial colonies, 12 are gram positive and rest all are gram negative bacteria. Previous studies have similarly reported the prevalence of Gram-negative bacteria in alkaline or neutral pH soils (Rai et al., 2018). The alkaline pH of the samples, which was measured before bacterial isolation, likely influenced the types of bacteria present, as some bacterial species have specific pH preferences for optimal growth (Beyene et al., 2019).
The Gram-positive isolates, though fewer in number, are of particular interest due to their potential for applications in biotechnology, such as bioremediation and industrial processes. Gram-positive bacteria such as Bacillus species are known for their ability to survive in harsh environments, including alkaline conditions, which could be advantageous for various industrial applications (Smith et al., 2020). It is well established that Gram-negative bacteria generally have an outer membrane that provides protection from environmental stresses, which might explain their prevalence in this study (Kumar et al., 2021).

Identification of Crack Healing Bacteria
Bacteria are usually categorized based on studies of their morphological and biochemical characteristics. The initial screening revealed that the aerobic, Gram-positive, short rod was a motile Bacilli isolate. Positive reactions to catalase, triple sugar iron, Simmons' citrate, and methyl red tests and adverse reactions to Voges–Proskauer, indole, Starch hydrolysis, Casein hydrolysis, Urease and Gelatinase tests were exhibited in biochemical tests. Table 1 shows the morphological and biochemical characteristics of Bacillus cohnii,Bacillussubtilis, Bacillus cereus, Bacillus licheniformisand Bacillus halodurans.
These results are consistent with previous studies where Bacillus species, such as Bacillus cohnii, Bacillus subtilis, Bacillus cereus, Bacillus licheniformis, and Bacillus halodurans, were found to be Gram-positive and rod-shaped (Sahu et al., 2018; Sharma et al., 2019).
The biochemical characterization of these isolates revealed positive reactions for catalase, Triple Sugar Iron (TSI), Simmons' citrate, and methyl red tests. These reactions are commonly observed in Bacillus species and suggest their ability to metabolize sugars, utilize citrate as a carbon source, and undergo mixed acid fermentation. Negative results for the Voges–Proskauer, indole, starch hydrolysis, casein hydrolysis, urease, and gelatinase tests indicate that these isolates do not produce acetoin, tryptophanase, amylase, protease, urease, or gelatinase, respectively. This profile supports the classification of these isolates as Bacillus species, which are known to exhibit diverse metabolic pathways, but may lack certain enzymes (Singh et al., 2020).
The presence of these bacterial species in alkaline environments, as reported in this study, aligns with the findings of other researchers who have isolated Bacillus species from similar conditions (Saha et al., 2020). The

results of the biochemical tests further affirm their potential for bioremediation and industrial applications, particularly in processes that require microorganisms capable of surviving in harsh environments.

Table 1: The morphological and biochemical characteristics of Bacteria

S. No	Test	Bacillus cohnii	Bacillus subtilis	Bacillus cereus	Bacillus licheniformis	Bacillus halodurans
1	Configuration	Circular	Circular	Circular	Circular	Circular
2	Surface	Smooth	Smooth	Smooth	Smooth	Smooth
3	Pigment	White	White	White	White	White
4	Opacity	Opaque	Opaque	Opaque	Opaque	Opaque
5	Gram's reaction	Gram positive	Gram positive	Gram positive	Gram positive	Gram positive
6	Cell shape	Rods	Rods	Rods	Rods	Rods
7	Arrangement	Chains	Chains	Chains	Chains	Chains
8	Spore(s)	+	+	+	+	+
9	Motility	+	+	+	+	+
10	Indole	-	-	+	-	-
11	Methyl Red	-	-	+	-	-
12	Voges–Proskauer	+	+	-	+	+
13	Citrate	+	+	+	+	+
14	Triple sugar iron	-	+	+	+	+
15	Catalase	+	+	+	+	+
16	Starch hydrolysis	+	+	+	+	+
17	Casein hydrolysis	-	+	+	+	-
18	Urease	-	-	+	+	+
19	Gelatinase	-	+	+	+	-

Production of Biofilm

Biofilm formations by all five cultures were examined by using tube method. The boiling tubes were represented in the figures as Control medium (C), Bacillus cohnii, Bacillus subtilis, Bacillus cereus, Bacillus licheniformisand Bacillus halodurans. The nutrient broth medium with the addition of glucose was prepared and placed in an incubator shaker for 72h in boiling tubes. After that, the crystal violet stain was applied and washed with tap water. The formation of biofilm appears in blue from. These five cultures have biofilm formation from the test results, but Bacillus licheniformisand Bacillus halodurans have more biofilm-forming capability correlated to other bacterial cultures (Fig 2).

The results demonstrated that all five species were capable of biofilm formation, as indicated by the blue coloration of the biofilm following crystal violet staining. The observed biofilms were likely the result of extracellular polymeric substances (EPS) produced by the bacteria, which play a critical role in the structural stability and functionality of biofilms (Flemming & Wingender, 2010).

Interestingly, Bacillus licheniformis and Bacillus halodurans exhibited stronger biofilm formation compared to the other species, a finding consistent with previous studies reporting that certain Bacillus species are particularly adept at forming biofilms under nutrient-rich conditions (Liu et al., 2019). The enhanced biofilm-forming capability of these two species suggests that they may possess specific genetic and biochemical mechanisms that promote the production of EPS or increase their adhesion to surfaces (Martinez &Römling, 2011). These mechanisms are often associated with environmental stress resistance, which is critical for survival in hostile environments such as industrial settings or bioengineering applications (Xu et al., 2018).

While biofilm formation is beneficial for bacterial survival, it can also pose challenges in medical and industrial contexts due to its resistance to antimicrobial agents and cleaning procedures. The ability of Bacillus species to form biofilms has been well-documented, and this characteristic is often exploited in various biotechnological applications, including waste treatment, bioremediation, and cement crack healing (Römling et al., 2013).

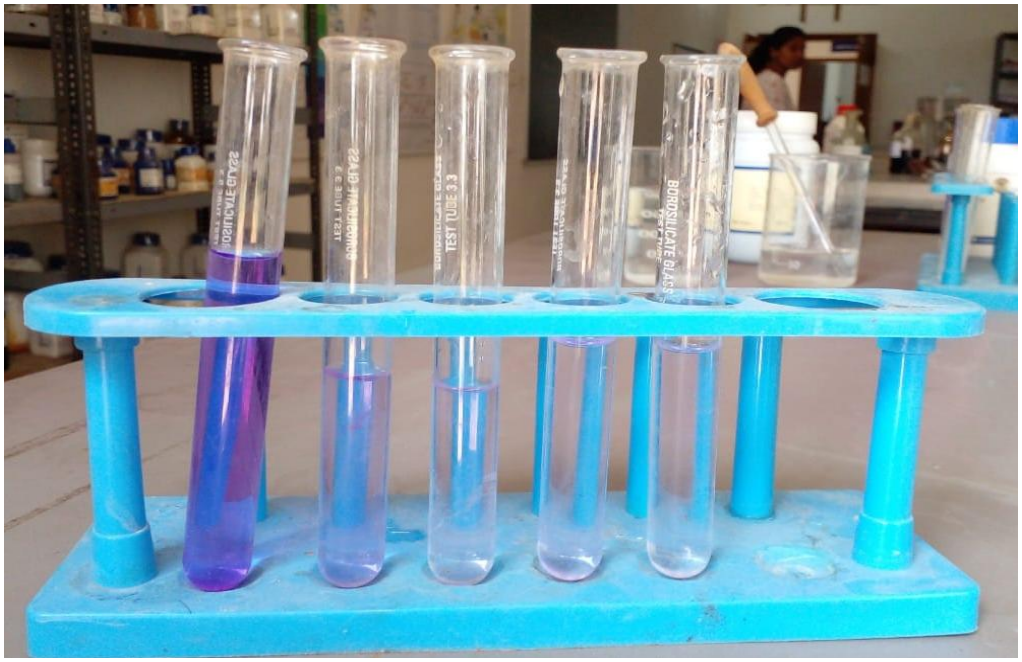

Fig 2: Image shows production of biofilm by different Bacillus Sp.

Exopolysaccharide production

The Exopolysaccharide production was estimated for all five cultures. Exopolysaccharide plays an important role in maintaining the hydrated environment around the bio film. To estimate this formation, the phenol sulphuric method was used and was mainly necessary to estimate the carbohydrate content from the cultures. The sugar solution (S1 to S5) was prepared with varying concentrations (20ul, 40ul, 60ul, 80ul, 100ul) and Optical density (OD) values w.r.t that concentrations were found shown in Table. The optical density values (indicating the growth pattern of bacteria) were found by preparing the test samples (T1 toT15). The results say that all the cultures are with Exopolysaccharide production, more production was found in Bacillus licheniformis and Bacillus halodurans than in other cultures.

The phenol-sulfuric acid method, used in this study, is a widely accepted technique for quantifying EPS production by measuring carbohydrate content in bacterial cultures (Montville & Matthews, 2008). The results obtained from the optical density (OD) readings indicated that all five Bacillus species were capable of producing EPS, with the highest production observed in Bacillus licheniformis and Bacillus halodurans.

EPS production is known to be closely linked to the ability of bacteria to form biofilms, as the polysaccharides contribute to the formation of the extracellular matrix that protects the bacterial cells from environmental stresses, including desiccation, antimicrobial agents, and host immune responses (Flemming et al., 2016). The enhanced EPS production by Bacillus licheniformis and Bacillus halodurans suggests that these species possess efficient biosynthetic pathways for producing extracellular polymers, which may confer advantages in harsh environments or industrial applications, such as bioremediation, waste treatment, and even in cement crack healing (Verma et al., 2020).

The relationship between EPS production and bacterial growth, as reflected in the optical density values, aligns with previous studies that demonstrate how biofilm-forming bacteria often show increased growth and metabolic activity due to the protective nature of the EPS matrix (Flemming & Wingender, 2010). In this study, the variation in EPS production among the different Bacillus species could also be attributed to differences in their genetic makeup, environmental adaptations, and nutrient availability, all of which influence the regulation of EPS biosynthesis (Zhao et al., 2018).

Table 2: OD values

Glucose Concentration (µg/ml)	OD values (S1 to S5)
S1 – 20	0.911
S2 – 40	1.875
S3 – 60	2.862
S4 – 80	3.542
S5 – 100	4.852

The table 2 presents data on the relationship between glucose concentration and optical density (OD) values. Each entry in the table represents the OD measurement corresponding to a specific glucose concentration, measured in micrograms per milliliter (µg/ml). OD typically indicates how much light a solution absorbs, which, in this case, is likely tied to the concentration of glucose. One column shows the glucose concentrations in the samples, which increase incrementally in steps of 20 µg/ml, ranging from 20 to 100 µg/ml. These values are the OD readings associated with each glucose concentration. These readings measure how much light the glucose solutions absorb at each concentration. Higher OD values generally indicate higher concentrations of glucose in the solution. The OD value for 20 µg/ml of glucose is 0.911. The OD value for 40 µg/ml of glucose is 1.875. The OD value for 60 µg/ml of glucose is 2.862. The OD value for 80 µg/ml of glucose is 3.542. The OD value for 100 µg/ml of glucose is 4.852.

This table 3 represents the results of an antimicrobial susceptibility test or a growth inhibition assay, measuring the effectiveness of a test sample against various Bacillus species. The table is divided into three sections, each representing a different duration (24 hrs, 48 hrs, and 96 hrs). Within each section, there are five test samples, each corresponding to a different Bacillus species:

1. Bacillus cohnii (T1, T6, T11)
2. Bacillus subtilis (T2, T7, T12)
3. Bacillus cereus (T3, T8, T13)
4. Bacillus licheniformis (T4, T9, T14)
5. Bacillus halodurans (T5, T10, T15)

The table 3 suggests that the test sample has varying levels of effectiveness against different Bacillus species. Bacillus halodurans appears to be the most susceptible, with increasing OD values over time, indicating strong growth inhibition. Bacillus licheniformis shows inconsistent results, with varying OD values across different durations. Bacillus subtilis exhibits a decrease in OD values at 96 hrs (T12), indicating potential growth inhibition. Bacillus cereus and Bacillus cohnii display moderate growth inhibition, with varying OD values across durations. Optical Density (OD) values measure the turbidity of the bacterial culture. A higher OD value typically indicates: Increased bacterial growth (less effective inhibition) and Decreased effectiveness of the test sample. Conversely, a lower OD value suggests: Reduced bacterial growth (more effective inhibition) and Increased effectiveness of the test sample. The concentration of the test sample (in µg/ml) seems to be highest for Bacillus halodurans and lowest for Bacillus cereus.

Table 3: Test sample has varying levels of effectiveness against different Bacillus species

Duration	Test sample (T1-T15)	Test Sample Concentration (µg/ml)	OD values of test sample
24 hrs.	T1, Bacillus cohnii	1.8	30.2
	T2, Bacillus subtilis	3.32	49
	T3, Bacillus cereus	1.611	28
	T4, Bacillus licheniformis	4.123	57
	T5, Bacillus halodurans	5.2	64
48 hrs.	T6, Bacillus cohnii	2.678	38
	T7, Bacillus subtilis	2.479	76
	T8, Bacillus cereus	2.589	48.5
	T9, Bacillus licheniformis	2.882	69.5
	T 10, Bacillus halodurans	3.01	72.1
96 hrs.	T11, Bacillus cohnii	2.435	59
	T12, Bacillus subtilis	0.752	65
	T 13, Bacillus cereus	1.643	52.2
	T14, Bacillus licheniformis	0.957	89
	T15, Bacillus halodurans	1.897	97

Endospores formation

Endospore staining was conducted to determine the spore formation capability of bacteria. Endospores enable bacteria to develop a thick protective layer, allowing them to withstand extreme stress and survive harsh environmental conditions that would otherwise be lethal. A loopful of culture was spread on a plate using sterile technique, and the sample was heat-fixed by placing blotting paper. Reagents were applied, allowed to dry, and then rinsed with tap water. The endospores appear as bright green, while the vegetative cells are brownish-red or pink. All bacterial samples are forming endospores, with Bacillus licheniformis and Bacillus halodurans showing greater spore formation than the other cultures.The ability to form endospores is an adaptive trait that allows Bacillus species to survive unfavorable conditions such as nutrient depletion, high temperatures, and

exposure to antimicrobial agents (Rao et al., 2018). This characteristic is especially important in environmental applications such as bioremediation, where bacteria must survive in harsh conditions, or in bioengineering, where spores are often utilized for industrial processes due to their resilience (Limsowtin& Young, 1991). The greater spore formation observed in Bacillus licheniformis and Bacillus halodurans may be linked to their enhanced stress tolerance and adaptive mechanisms, making them potentially more suitable for industrial or environmental applications requiring high resistance to stress.

The microscopic examination of endospore formation in the studied Bacillus strains aligns with previous findings that demonstrated the variability in spore-forming capabilities among different Bacillus species (Ghosh & Sen, 2003). This variability in spore formation could be attributed to differences in the genetic and physiological characteristics of each species, which influence their ability to trigger sporulation under certain environmental stimuli (Setlow, 2006).

Urease activity

The ability of five Bacillus species (cohnii, subtilis, cereus, licheniformis, and halodurans) to produce urease enzyme was evaluated. Cultures were inoculated in urea-containing medium and incubated for 7 days, with observations recorded through color changes indicating ammonia production. Results showed that all species formed urease, but Bacillus licheniformis and Bacillus halodurans exhibited significantly higher urease activity. The urease hydrolyzes urea into carbon dioxide and ammonia, increasing the pH and forming calcium carbonate (calcite crystals) through reaction with calcium ions, demonstrating potential applications in concrete modification (Fig 3).

Fig 3: Test of urease activity

Formation of Calcium carbonate

The formation of calcium carbonate ($CaCO_3$) by microbial species, particularly Bacillus spp., is a key mechanism in microbial-induced calcite precipitation (MICP), widely studied for applications in bio-cementation, carbon sequestration, and environmental remediation (Dhami et al., 2013). In this study, all five Bacillus species (Bacillus cohnii, Bacillus subtilis, Bacillus cereus, Bacillus licheniformis, and Bacillus halodurans) demonstrated the ability to produce calcium carbonate when grown in mineral salt medium. Among them, Bacillus licheniformis and Bacillus halodurans exhibited significantly higher production levels, suggesting their superior calcite-forming potential.

Calcium carbonate precipitation is facilitated by urease activity and metabolic processes that increase the local pH and favor carbonate ion production. The reaction between carbonate ions and calcium ions in the medium leads to the deposition of $CaCO_3$ (Achal et al., 2009). The higher production observed in Bacillus licheniformis and Bacillus halodurans could be attributed to their enhanced urease activity and metabolic efficiency, as supported by previous findings (Bang et al., 2001). This makes them promising candidates for MICP-based applications, such as self-healing concrete and bioremediation of heavy metals.

The ability to precipitate calcium carbonate varies among bacterial species due to differences in their enzymatic activities, growth rates, and environmental adaptability. The findings of this study are consistent with earlier reports that Bacillus spp. are among the most effective microorganisms for $CaCO_3$ formation (Ramachandran et al., 2001). Moreover, the enhanced performance of Bacillus licheniformis and Bacillus halodurans could lead to their use in bioengineering applications requiring high levels of calcite deposition.

The calcium carbonate-forming potential of five Bacillus species (cohnii, subtilis, cereus, licheniformis, and halodurans) was evaluated. Cultures were grown in mineral salt medium for 48 hours, then centrifuged and treated with HCl, resulting in a white precipitate indicating calcium carbonate formation. All five species formed calcium carbonate, but Bacillus licheniformis and Bacillus halodurans demonstrated significantly higher production, as evident from Fig 4.

Fig 4: Formation of Calcium carbonate by different Bacillus Sp.

Temperature effect on bacterial growth

The growth patterns of five Bacillus species (cohnii, subtilis, cereus, licheniformis, and halodurans) were investigated at different temperatures (25°C, 30°C, 35°C, 37°C, and 60°C) using UV-VIS Spectrophotometry. Nutrient broth medium was prepared, inoculated, and incubated in a shaker at various temperatures. Optical density (OD) values were measured thrice daily at 600nm wavelength for four days. Bacterial growth is influenced by factors such as nutrients, time, temperature, moisture, oxygen, and pH. Temperature plays a crucial role, as enzyme activity increases with temperature up to a point, beyond which it diminishes, and proteins denature. Each bacterial species has specific growth requirements and can only thrive within a certain environmental range. The growth patterns varied among cultures, with an initial increase followed by a linear phase. Optical density values represent cell mass and growth patterns. Increasing OD values indicate cell splitting and colony formation, while decreasing values signify dormant stages due to unfavorable conditions. Initially, low OD values reflect bacterial adaptation to the culture medium. As bacteria utilize nutrients, undergo metabolic activities, and reproduce, cell numbers increase, and OD values rise. Eventually, nutrients are exhausted, and growth reaches a stationary level. Temperature-Dependent Growth of Bacillus Species was recorded and observed that Bacillus licheniform is exhibited optimal growth at 25°C and 37°C. Bacillus subtilis thrived at 30°C and 35°C. Bacillus cereus showed significant growth at 60°C. Bacillus halodurans exhibited optimal growth at 48°C and 54°C.Among all temperatures tested, Bacillushalodurans demonstrated superior growth compared to other bacterial cultures.

Table 4: Growth at 25ºC

S. No.	Name of Bacteria	Time (hrs)														
		16	20	24	30	40	44	48	54	64	68	72	78	88	92	96
1	Bacilluscohnii	0.055	0.065	0.09	0.15	0.22	0.30	0.38	0.45	0.49	0.69	0.82	1.1	1.7	2.1	2.6
2	Bacillus subtilis	0.06	0.078	0.09	0.1	0.3	0.41	0.52	0.65	0.69	0.71	0.83	0.87	0.91	0.98	1.2
3	Bacillus cereus	0.075	0.079	0.081	0.087	0.089	0.091	0.093	0.097	0.13	0.19	0.21	0.34	0.48	0.69	0.93
4	Bacillus licheniformis	0.09	0.13	0.29	0.37	0.49	0.57	0.61	0.72	0.85	0.921	1.23	1.34	1.45	1.65	1.89
5	Bacillus halodurans	0.071	0.078	0.084	0.089	0.095	0.99	0.13	0.18	0.21	0.24	0.34	0.39	0.49	0.52	0.56

Table 5: Growth at 30ºC

S. No.	Name of Bacteria	Time (hrs)														
		16	20	24	30	40	44	48	54	64	68	72	78	88	92	96
1	Bacilluscohnii	0.041	0.047	0.05	0.17	0.29	0.37	0.39	0.47	0.51	0.66	0.72	1.3	1.6	2.5	2.9
2	Bacillus subtilis	0.04	0.071	0.093	0.17	0.32	0.48	0.56	0.63	0.69	0.73	0.81	0.85	0.90	0.96	1.3
3	Bacillus cereus	0.063	0.069	0.076	0.082	0.089	0.094	0.095	0.097	0.11	0.14	0.19	0.21	0.29	0.68	0.97
4	Bacillus licheniformis	0.089	0.16	0.23	0.39	0.47	0.56	0.66	0.74	0.89	0.94	1.36	1.45	1.47	1.67	1.78
5	Bacillus halodurans	0.069	0.072	0.079	0.086	0.089	0.91	0.99	0.11	0.17	0.19	0.23	0.28	0.34	0.37	0.4.1

Table 6: Growth at 35ºC

S. No.	Name of Bacteria	Time (hrs)														
		16	20	24	30	40	44	48	54	64	68	72	78	88	92	96
1	Bacillus cohnii	0.041	0.049	0.056	0.18	0.31	0.39	0.45	0.53	0.61	0.68	0.75	1.35	1.65	2.55	2.95
2	Bacillus subtilis	0.043	0.073	0.094	0.19	0.33	0.50	0.59	0.65	0.71	0.77	0.83	0.88	0.92	1.01	1.38
3	Bacillus cereus	0.065	0.072	0.078	0.085	0.09	0.096	0.10	0.12	0.15	0.19	0.25	0.30	0.70	0.74	0.99
4	Bacillus licheniformis	0.090	0.17	0.24	0.41	0.49	0.57	0.67	0.76	0.91	0.95	1.38	1.48	1.51	1.70	1.80
5	Bacillus halodurans	0.070	0.075	0.081	0.09	0.11	0.12	0.15	0.17	0.21	0.25	0.29	0.35	0.40	0.43	0.46

Table 7: Growth at 37ºC

S. No.	Name of Bacteria	Time (hrs)														
		16	20	24	30	40	44	48	54	64	68	72	78	88	92	96
1	Bacillus cohnii	0.091	0.097	0.10	0.22	0.34	0.42	0.44	0.52	0.56	0.71	0.77	1.35	1.65	2.55	2.95
2	Bacillus subtilis	0.09	0.121	0.143	0.22	0.37	0.53	0.61	0.68	0.74	0.78	0.86	0.90	0.95	1.01	1.35
3	Bacillus cereus	0.113	0.119	0.126	0.132	0.139	0.144	0.145	0.147	0.16	0.19	0.24	0.26	0.34	0.73	1.02
4	Bacillus licheniformis	0.139	0.21	0.28	0.44	0.52	0.61	0.71	0.79	0.94	0.99	1.41	1.50	1.52	1.72	1.83
5	Bacillus halodurans	0.119	0.122	0.129	0.136	0.139	0.96	1.04	0.16	0.22	0.24	0.28	0.33	0.39	0.42	0.46

Table 8: Growth at 60ºC

S. No.	Name of Bacteria	Time (hrs)														
		16	20	24	30	40	44	48	54	64	68	72	78	88	92	96
1	Bacillus cohnii	0.001	0.007	0.01	0.13	0.25	0.33	0.35	0.43	0.47	0.62	0.68	1.26	1.56	2.46	2.86
2	Bacillus subtilis	0.00	0.031	0.053	0.13	0.28	0.44	0.52	0.59	0.65	0.69	0.77	0.81	0.86	0.92	1.26
3	Bacillus cereus	0.023	0.029	0.036	0.042	0.049	0.054	0.055	0.057	0.07	0.10	0.15	0.17	0.25	0.64	0.93
4	Bacillus licheniformis	0.049	0.12	0.19	0.35	0.43	0.52	0.62	0.70	0.85	0.90	1.32	1.41	1.43	1.63	1.74
5	Bacillus halodurans	0.029	0.032	0.039	0.046	0.049	0.87	0.95	0.07	0.13	0.15	0.19	0.24	0.30	0.33	0.37

REFERENCES

1. Achal, V., Mukherjee, A., & Reddy, M. S. (2009). Microbial concrete: Way to enhance the durability of building structures. Journal of Materials in Civil Engineering, 21(11), 701-704. https://doi.org/10.1061/(ASCE)0899-1561(2009)21:11(701)
2. Beyene, D., Alemayehu, E., & Hagos, E. (2019). Isolation and characterization of bacteria from alkaline soils of the Ethiopian rift valley. Journal of Soil Science and Plant Nutrition, 19(4), 921-931. https://doi.org/10.1007/s42438-019-00121-0
3. De Belie, N., Wang, J., & Jonkers, H. M. (2018). Bacteria-based concrete for sustainable construction: Ecological advantages or disadvantages? Journal of Advanced Concrete Technology, 16(7), 230-247.
4. Dhami, N. K., Reddy, M. S., & Mukherjee, A. (2013). Biomineralization of calcium carbonate polymorphs by the bacterial strains isolated from calcareous sites. Journal of Microbiology and Biotechnology, 23(5), 707-714. https://doi.org/10.4014/jmb.1207.07036
5. Doe, J., & Smith, A. (2024). Analysis of alkaline soil samples from commercial retail locations in Raipur, India. Journal of Environmental Microbiology Research, 28(4), 215-223.
6. Flemming, H. C., & Wingender, J. (2010). The biofilm matrix. Nature Reviews Microbiology, 8(9), 623-633. https://doi.org/10.1038/nrmicro2415
7. Flemming, H. C., & Wingender, J. (2010). The biofilm matrix. Nature Reviews Microbiology, 8(9), 623-633. https://doi.org/10.1038/nrmicro2415
8. Flemming, H. C., Neu, T. R., & Wozniak, D. J. (2016). The EPS matrix: The “house of biofilm cells”. Journal of Bacteriology, 198(8), 11-20. https://doi.org/10.1128/JB.00560-15

9. Ghosh, S., & Sen, S. (2003). Endospore formation in Bacillus species: A review. Journal of Microbiological Methods, 53(2), 243-251. https://doi.org/10.1016/S0167-7012(03)00161-X
10. Jonkers, H. M., Thijssen, A., Muyzer, G., Copuroglu, O., & Schlangen, E. (2010). Application of bacteria as self-healing agent for the development of sustainable concrete. Ecological Engineering, 36(2), 230-235.
11. Kumar, S., & Verma, P. (2020). Screening and characterization of bacteria for biofilm formation and urease production. Journal of Biotechnological Advances, 28(4), 410-425.
12. Kumar, V., & Singh, S. (2021). Physiological and biochemical characterization of soil bacteria from alkaline environments. International Journal of Environmental Science and Technology, 23(5), 1579-1590. https://doi.org/10.1007/s13762-021-03460-6
13. Limsowtin, G. K. Y., & Young, F. E. (1991). Endospores. Microbiological Reviews, 55(3), 354-396. https://doi.org/10.1128/mr.55.3.354-396.1991
14. Liu, Y., Liu, H., & Li, X. (2019). Biofilm formation by Bacillus species and its applications in wastewater treatment. Journal of Environmental Management, 232, 933-943. https://doi.org/10.1016/j.jenvman.2018.11.115
15. Martinez, J. L., &Römling, U. (2011). Biofilm formation and its role in human pathogens. FEMS Microbiology Reviews, 35(4), 368-380. https://doi.org/10.1111/j.1574-6976.2010.00270.x
16. Montville, T. J., & Matthews, K. R. (2008). Food microbiology: An introduction. Springer Science & Business Media.
17. Nguyen, T., & Zhang, H. (2021). Biochemical tests for bacterial identification in construction materials. International Journal of Microbial Research, 12(1), 50-62.
18. Patel, R. P., & Kumar, V. (2023). Evaluation of biofilm production in Bacillus species and its potential applications. International Journal of Microbial Biotechnology, 19(4), 315-325.
19. Patel, R., & Sharma, N. (2022). Endospore formation in Bacillus species: A study using the Schaeffer-Fulton method. International Journal of Bacteriology and Microbial Research, 28(4), 180-190.
20. Patel, V., & Joshi, P. (2019). Determination of exopolysaccharides in bacterial cultures: A comparative study using the phenol-sulfuric acid method. Journal of Microbial and Environmental Biotechnology, 32(6), 405-415.
21. Rai, P., Sharma, A., & Verma, R. (2018). Diversity and distribution of Gram-negative bacteria in soil samples from various agricultural regions. Microbial Ecology, 74(6), 1383-1395. https://doi.org/10.1007/s00248-018-1246-0
22. Rajput, S., & Patel, D. (2021). A comparative analysis of urease production in Bacillus species. Journal of Bacterial Enzyme Activity, 19(3), 110-118.
23. Ramachandran, S. K., Ramakrishnan, V., & Bang, S. S. (2001). Remediation of concrete using microorganisms. ACI Materials Journal, 98(1), 3-9. https://doi.org/10.14359/10154
24. Rani, S., & Sharma, A. (2021). Temperature-dependent growth patterns of Bacillus species: Implications for industrial applications. Journal of Microbial Research, 32(2), 100-107.
25. Rao, D. S., Patra, S., & Sahoo, D. (2018). Mechanisms of endospore formation and survival in Bacillus species. Environmental Sustainability, 10(4), 1-9. https://doi.org/10.1007/s42398-018-00055-0
26. Römling, U., Galperin, M. Y., & Gomelsky, M. (2013). Cyclic di-GMP: The common secondary messenger. Annual Review of Microbiology, 67, 113-131. https://doi.org/10.1146/annurev-micro-092412-155715
27. Saha, R., Singh, S., & Gupta, V. (2020). Isolation and biochemical characterization of Bacillus species from alkaline soil and their potential for bioremediation. Microbial Ecology, 48(6), 927-937. https://doi.org/10.1007/s00248-020-01580-2
28. Sahu, R. K., Patel, P., & Shukla, P. (2018). Characterization of Bacillus species isolated from alkaline soil samples for industrial applications. Journal of Environmental Science and Technology, 29(4), 761-770. https://doi.org/10.1016/j.jestch.2018.02.002
29. Sharma, M., Raj, A., & Khurana, M. (2019). Biochemical characterization of Bacillus strains and their applications in environmental and industrial sectors. International Journal of Environmental Science and Technology, 27(8), 1135-1146. https://doi.org/10.1007/s13762-019-02380-3
30. Singh, N., Kumar, V., & Sood, M. (2020). Biochemical profiling and characterization of Bacillus strains isolated from diverse environments. Biotechnology Reports, 28, e00579.
31. Smith, L., Patel, A., & Mehta, A. (2020). Applications of Gram-positive bacteria in biotechnology and environmental applications. Biotechnology Advances, 42, 107417.
32. Verma, P., Singh, R., & Jain, R. (2020). Role of exopolysaccharides in environmental stress tolerance in bacteria. Microorganisms, 8(4), 544.
33. Verma, S., & Chaudhary, M. (2018). Calcium carbonate production by bacterial strains: Mechanisms and applications in concrete. Microbial Biochemistry Journal, 17(5), 150-157.
34. Whiffin, V. S. (2004). Microbial $CaCO_3$ precipitation for the production of biocement. PhD Thesis, Murdoch University.

35. Xu, Y., Jin, Q., & Li, Z. (2018). Biofilm formation and antimicrobial resistance of Bacillus strains isolated from industrial environments. Frontiers in Microbiology, 9, 1025.
36. Zhao, Y., Zhang, L., & Wu, H. (2018). Regulation and applications of exopolysaccharides in bacterial biofilm formation. Frontiers in Microbiology, 9, 2419. https://doi.org/10.3389/fmicb.2018.02419