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Analysis and optimization of process parameters for *in vitro* biomineralization of $CaCO_3$ by *Klebsiella pneumoniae*, isolated from a stalactite from the Sahastradhara cave†

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Stalactite is a speleothem which is usually made up of calcium carbonate crystals. In the present study the bacterial isolates, recovered from a stalactite from the Sahastradhara cave, were screened for their ability to precipitate calcium carbonate in order to understand whether mineralization in caves is a biogenic process or not. Five bacterial isolates were found to precipitate calcium carbonate *via* urease. The most potent bacterial isolate was identified as *Klebsiella pneumoniae* (accession number MG946801) based on 16S rDNA sequencing. The optimized conditions, for calcium carbonate precipitation, determined by response surface methodology using CCD were found to be: 1.5625% urea, 19.98% inoculum level, 6.98 pH and 38 h 24 min. The morphology and crystalline structure of the precipitated mineral were revealed by SEM. EDX analysis confirmed the presence of carbon, oxygen and calcium in a precipitated crystal. XRD analysis confirmed the crystalline structure of a mineral with rhombohedral shape and 166 Å crystal size. This bacterium can serve as a promising candidate for producing bioconcrete.

1. Introduction

Calcium carbonate is the most important mineral on earth as it constitutes 4% of total earth crust. Calcite, aragonite and vaterite are pure forms of calcium carbonate which differ in structure.[4] Calcite is a most stable form of $CaCO_3$ with rhombohedral shape. The hydrated forms of minerals comprise about 60% of biogenic minerals such as the two forms of carbonate monohydrocalcite and one amorphous form containing water. Calcification is a general phenomenon among the bacteria but cave dwelling bacteria which lack photosynthetic activity, play a crucial role in calcium carbonate precipitation. However, there are still ambiguities regarding the origin of speleothems in caves. There are reports indicating that the formation of speleothems, such as stalactites and stalagmites through the precipitation of calcite, is an abiogenic process[10,29] while many workers have reported the crucial role of microorganisms in calcium carbonate precipitation during speleogenesis.[7,8,23,25,26,32]

The role of microorganisms in the cave environment can not be underestimated as studies have shown that microbial metabolism is responsible for mineral precipitation and dissolution of cave walls.[22] However, the existing knowledge about the life-forms and biogeochemical processes contained within them is meager primarily due to difficulties in approaching this habitat. A variety of precipitation processes in caves result in the deposition of carbonate speleothems, silicates, iron and manganese oxides, sulfur compounds and nitrates. Among all the speleothems, stalactites are the most frequent and most common and resemble carrots hanging from cave ceilings. In cave ecosystems, the varied heterotrophic microbial communities in stalactite are well documented *viz.*, *Bacillus* and *Streptomyces* from stalactite of Grotta dei Cervi,[30] *Kocuria* sp. from stalactite of Cervo cave,[12] *Bacillus pumilis* and *Bacillus thuringiensis* from stalactite of Sahastradhara cave,[8,9] *Bacillus*, *Burkholderia*, and *Pasteurella* spp. from Gypsum cave of grave grubbo,[13] *Arthrobacter* and *Rhodococcus* sp. from stalactite of Pristine Karstic Herrenberg cave.[35]

Stalactites are usually composed of calcite but may consist of other minerals. The calcium carbonate mineralization ability of cave bacteria has been widely reported *viz.*, *Rhodococcus* sp. from Grotta dei Cervi,[21] *Bacillus pumilis* and *B. thuringiensis* from Sahastradhara cave,[8] *Bacillus* sp. from cave of central China,[39] *Arthrobacter* and *Rhodococcus* sp. from Pristine Karstic Herrenberg cave,[35] *Lysinibacillus* sp. and *Bacillus* sp. from caves of Meghalaya[5] and *Bacillus subtilis* and *Cupriavidus* sp. from Rani cave.[16] Several bacteria of Enterobacteriaceae family have been reported to form a crystalline structure containing calcium.[28] The microorganisms precipitate calcium carbonate

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through various processes *viz.*, photosynthesis, ammonification, denitrification, carbonic anhydrase production, urease enzyme production. Calcium precipitation through urease enzyme activity has been widely studied because it generates carbonate more easily than other reactions.[1–3,18,19,27,36]

The present study was carried out to determine the *in vitro* potential of bacteria recovered from stalactite of Sahastradhara cave to understand the origin of stalactite in the cave. Sahastradhara cave is located along the bank of river Baldi in Dehradun valley, Uttarakhand, India. The various factors which can affect the calcium carbonate precipitation were optimized using Response Surface Methodology (RSM) as it is a powerful and efficient mathematical approach which has been widely applied in the optimization processes.[17,31,33] It helps in the understanding of the interaction between the factors affecting the response in fewer experimental trials which is not possible with the traditional one-factor-at-a-time approach.

Table 1 Coded and noncoded value of independent variables

Independent variable	Coded levels	−2	−1	0	1	2
Urea percentage (%)	X_1	0.5	1	1.5	2	2.5
Inoculum percentage (%)	X_2	10	15	20	25	30
pH	X_3	6	6.5	7	7.5	8
Time (h)	X_4	12	24	36	48	60

2. Experimental

2.1 Sampling

Stalactite samples were collected aseptically from the cave and carried to the laboratory in the ice bucket. The samples were air dried for 48 h and crushed into a fine powder using sterile mortar and pestle.

2.2 Isolation of calcium precipitating bacteria

Calcium precipitating bacteria of stalactite samples were recovered by enrichment culturing on B4 medium (2.5 g l^{-1} calcium acetate, 10 g l^{-1} glucose, 4 g l^{-1} yeast extract) at 30 °C for 10 days.

Table 2 Experimental response of the dependable variable upon calcium carbonate precipitation

	Coded variables				Uncoded variables				
Run	X_1	X_2	X_3	X_4	Urea concentration (%)	Inoculum level (%)	pH	Time (h)	Response (%) of $CaCO_3$ precipitated
1	2	0	0	0	2.5	20	7	36	97.453
2	2	0	0	0	2.5	20	7	36	97.562
3	0	0	0	0	1.5	20	7	36	99.765
4	0	0	0	0	1.5	20	7	36	99.632
5	0	0	0	0	1.5	20	7	36	99.542
6	−1	1	1	−1	1	25	7.5	24	96.763
7	−1	−1	−1	−1	1	15	6.5	24	96.342
8	−1	−1	1	−1	1	15	7.5	24	97.021
9	−1	1	−1	−1	1	25	6.5	24	95.923
10	−1	−1	−1	1	1	15	6.5	48	97.145
11	−1	1	1	1	1	25	7.5	48	96.231
12	−1	−1	1	1	1	15	7.5	48	96.873
13	−1	1	−1	1	1	25	6.5	48	96.321
14	0	0	0	0	1.5	20	7	36	99.365
15	0	0	0	0	1.5	20	7	36	99.674
16	0	0	0	0	1.5	20	7	36	99.567
17	1	1	−1	−1	2	25	6.5	24	93.563
18	1	−1	1	−1	2	15	7.5	24	93.432
19	1	−1	−1	1	2	15	6.5	48	93.564
20	1	−1	1	1	2	15	7.5	48	93.564
21	1	1	1	1	2	25	7.5	48	93.763
22	1	1	1	−1	2	25	7.5	24	93.932
23	1	1	−1	1	2	25	6.5	48	94.231
24	1	−1	−1	−1	2	15	6.5	24	91.674
25	0	0	0	0	1.5	20	7	36	99.598
26	0	0	0	0	1.5	20	7	36	99.819
27	0	0	0	0	1.5	20	7	36	99.879
28	−2	0	0	0	0.5	20	7	36	91.876
29	−2	0	0	0	0.5	20	7	36	91.743
30	0	0	2	0	1.5	20	8	36	92.543
31	0	2	0	0	1.5	30	7	36	92.986
32	0	0	0	−2	1.5	20	7	12	92.764
33	0	0	0	2	1.5	20	7	60	94.682
34	0	0	−2	0	1.5	20	6	36	94.419
35	0	−2	0	0	1.5	10	7	36	94.862

2.3 Screening for calcium precipitation

2.3.1. Qualitative screening. All bacterial isolates recovered were screened for their ability to precipitate calcium either by production of carbonic anhydrase or urease. Carbonic anhydrase production was estimated by *p*-NPA (para nitro phenyl acetate) method.[34] Urease production was determined on urea agar medium as described by Cappuccino and Sherman.[15]

Fig. 1 Stalactite sample of Sahastradhara cave.

2.3.2. Quantitative estimation. The bacterial isolates were inoculated in B4 medium containing 1% urea. pH measurement and calcium carbonate estimation was done at an interval of 12 h up to 60 h. The concentration of Ca^{2+} was measured by EDTA titration method.[31] Mass of calcium was calculated using the following formula:

$$\text{Millimoles of EDTA} = \text{ml EDTA} \times \text{molarity EDTA} = \text{millimoles of calcium}$$

$$\text{Mass of calcium} = \text{millimoles of calcium} \times \text{molar mass of calcium (40.08)}$$

2.4 Optimization of calcium carbonate precipitation

The precipitation of calcium carbonate by the best isolate was optimized through response surface methodology using Design Expert 10 software (Stat-Ease Inc., Minneapolis, USA). Central Composite Design was used to generate a quadratic response with four factors, *viz.*, urea percentage (X_1), inoculum percentage (X_2), pH (X_3), time (X_4). The coded and non-coded (actual) level of the variables is given in Table 1. The central composite rotatable design for experimental runs and combinations for biomineralization is given in Table 2. A total of 35 runs were performed in triplicate.

2.5 Morphological and elemental analysis

The morphology of stalactite samples collected from both caves and precipitated calcium carbonate crystal was done by scanning electron microscopy (SEM) and X-ray diffraction (XRD)

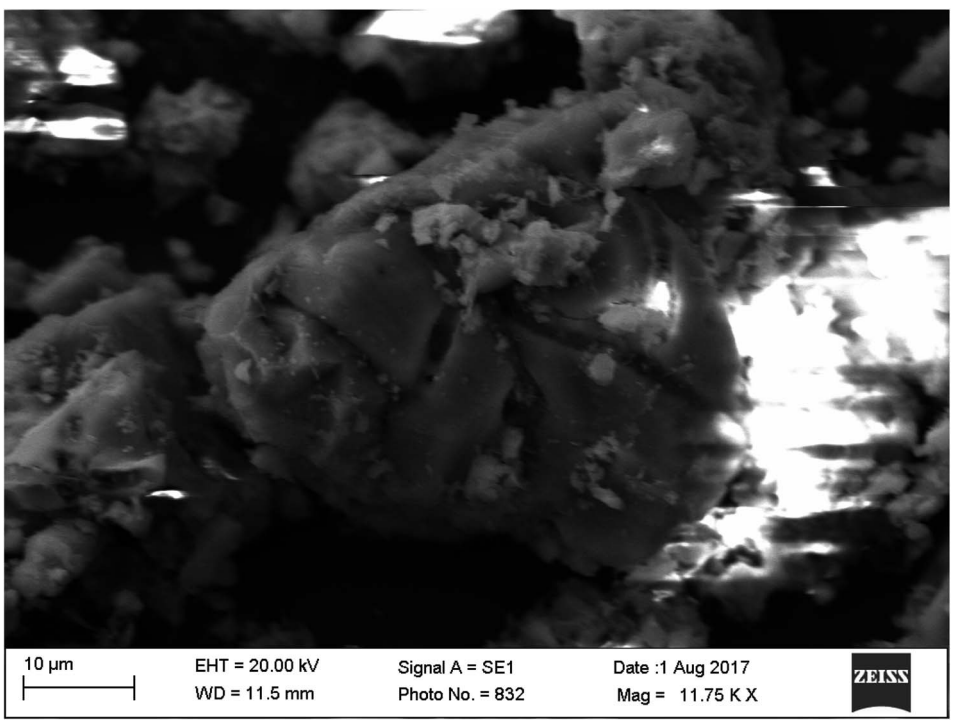

Fig. 2 Morphology of stalactite sample collected from Sahastradhara cave.

analysis. The elemental composition was determined by Energy Dispersive X-ray (EDX) analysis.

2.6 Identification of calcium precipitating bacteria

The phylogenetic analysis of the most potent calcium precipitating bacterial isolate was done by 16S rDNA sequencing using PCR amplification primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′). The sequencing was done using primers 907R (5′-CCGTCAATTCCTTTRAGTTT-3′) and 785F (5′-GGATTAGATACCCTGGTA-3′) and BDT v3.1 cycle sequencing kit on ABI 3730xl genetic analyzer. The obtained sequences were used to carry out BLAST analysis with the NCBI gene bank database. Based on the maximum identity score, first ten sequences were selected and aligned using Clustal W. Phylogenetic tree was constructed using MEGA 7.0. The sequence was then submitted to NCBI database for procurement of accession number.

3. Results and discussion

3.1 Analysis of stalactite

The crystalline structure of stalactite of Sahastradhara cave (Fig. 1) matched with calcium magnesium carbonate belonging to rhombohedral crystal system (reference code: 00-060-0473; Fig. 2). The stalactite sample of Sahastradhara cave was found to contain 29.41% carbon, 46.89% oxygen, 23.25% calcium and 0.45% magnesium (Fig. 3). The percentage of carbon was found to be quite high as compared to that reported in stalactite of Lake cave (4.9 ± 1.7%) and Mammoth cave (5.4 ± 1.5%) as reported by Dhami *et al.*[40] They reported the presence of many other elements like N, Si, K, Na, S, Mg, Al, Fe and P which were not detected in stalactite of Sahastradhara cave except magnesium.

3.2 Estimation of calcium carbonate precipitation

A total of 6 bacterial isolates were found to produce urease (Fig. S1†). None of the isolate produced carbonic anhydrase. Urea hydrolysis generates carbonate most easily than any other reactions. If the urease producing microorganism is present in calcium-rich environment, then the precipitation of calcium carbonate occurs.

B4SSt3 was found to carry out the maximum precipitation (97%) of calcium carbonate raising the pH of medium to 9.0 ± 0.05 (Table 3 and Fig. S2†). The rise in pH of medium can be due to higher urease activity as reported by Cabrera *et al.*[11] and Okyay and Rodrigues.[33] No other bacterial isolate could match with B4SSt3. As there was only a marginal increase in calcium precipitation from 48 h to 60 h, therefore further experiment was not carried out.

3.3 Identification of calcium precipitating bacteria

The isolate B4SSt3 was identified to be *Klebsiella pneumoniae* based on 16S rDNA sequence analysis (Fig. S3†). The sequence was submitted to NCBI nucleotide database with accession number MG946801.

The presence of *Klebsiella pneumoniae* in the cave has been observed by few researchers. Campbell *et al.*[14] reported *Klebsiella ozaenae*, *K. oxytoca* and *K. pneumoniae* in Pettyjohns cave. Seman *et al.*[38] reported *Klebsiella ornithinolytica* and *K. oxytoca* from Domica and Gombasecka cave, *Klebsiella ozaenae* from Domica and Milada cave and *Klebsiella pneumoniae* from Krasnohorska cave. The exploration of ammonia oxidation (amoA) and nitrogen fixation gene in Lava caves of Terceira, Azores and Portugal revealed that the nitrogen fixation community was dominated by *Klebsiella pneumoniae*-like sequences.[24] Urease production from *Klebsiella pneumoniae* has also been well studied.[20] However, no report of biomineralization potential of *Klebsiella pneumoniae* has been reported from caves. Till date, the calcium carbonate precipitation studies of cave have been limited to *Bacillus*, *Cupriavidus* sp., *Lysinibacillus* sp. and actinomycetes only.[5,8,9,16,37] Baskar *et al.*[9] identified *Bacillus anthracis*, *B. cereus*, *B. circulans*, *B. lentus*, *B. pumilis*, *B. sphaericus* and Actinomycetes as calcium precipitating isolates from moonmilk and stalactite sample of Sahastradhara cave.

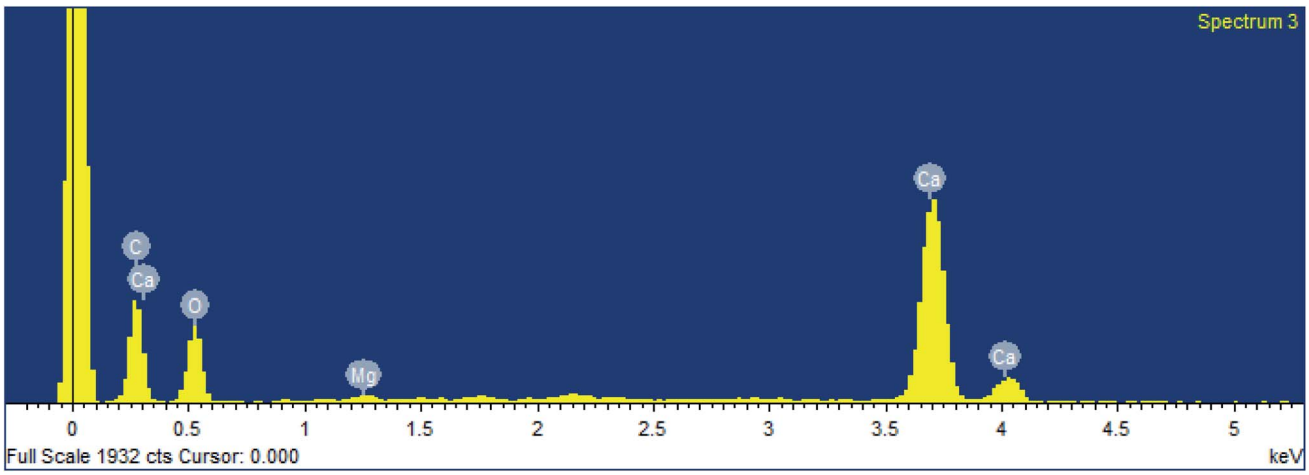

Fig. 3 EDX analysis of stalactite sample collected from Sahastradhara cave.

Table 3 Amount (%) of calcium carbonate precipitated by the urease positive bacterial isolates[a]

Name of bacterial isolate	Initial pH	12 h		24 h		36 h		48 h		60 h	
		pH	$CaCO_3$ (%)	pH	$CaCO_3$ (%)	pH	$CaCO_3$ (%)	pH	$CaCO_3$ (%)	pH	$CaCO_3$ (%)
B4SSt1	6.00 ± 0.00	6.00 ± 0.02	12.13 ± 0.05	6.00 ± 0.01	13.50 ± 0.05	6.00 ± 0.01	14.32 ± 0.05	6.00 ± 0.05	15.89 ± 0.08	6.00 ± 0.02	16.32 ± 0.09
B4SSt2	6.00 ± 0.00	6.00 ± 0.01	10.67 ± 0.03	8.00 ± 0.04	19.00 ± 0.02	8.00 ± 0.10	24.08 ± 0.12	8.00 ± 0.10	26.32 ± 0.10	8.00 ± 0.04	26.63 ± 0.10
B4SSt3	6.00 ± 0.00	6.00 ± 0.01	78.00 ± 0.02	9.00 ± 0.01	87.00 ± 0.01	9.00 ± 0.11	92.00 ± 0.23	9.00 ± 0.11	96.00 ± 0.21	9.00 ± 0.05	97.00 ± 0.23
B4SSt4	6.00 ± 0.00	6.00 ± 0.05	15.38 ± 0.15	6.50 ± 0.02	18.45 ± 0.05	7.00 ± 0.08	25.64 ± 0.12	7.00 ± 0.11	26.43 ± 0.08	7.00 ± 0.10	27.87 ± 0.12
B4SSt5	6.00 ± 0.00	6.00 ± 0.02	12.43 ± 0.60	6.00 ± 0.01	14.32 ± 0.02	7.00 ± 0.10	20.32 ± 0.14	7.00 ± 0.08	21.03 ± 0.10	7.00 ± 0.12	22.47 ± 0.10

[a] Each value is expressed as mean value. B4 – B4 medium; SSt – Sahastradhara stalactite.

Table 4 Determination of significance of regression model by ANOVA[a]

Source	Term degree of freedom	Error degree of freedom	F	Prob > F
Whole-plot	4	1.09	294.141	0.03454
X_1	1	2.9	1067	<0.0001
X_1^2	1	2.53	2075	<0.001
$X_1X_2^2$	1	1.37	1358.43	0.00505
$X_1^2X_2^2$	1	1.19	108.425	0.0407
Subplot	20	4.62	192.09	<0.0001
X_2	1	4.62	111.53	<0.0001
X_3	1	8	112.06	<0.0001
X_4	1	8	116.83	<0.0001
X_1X_2	1	8	114.77	<0.0001
X_1X_3	1	8	1.11	0.322
X_1X_4	1	8	16.17	0.0038
X_2X_3	1	8	9.28	0.0159
X_2X_4	1	8	21.2	0.0017
X_3X_4	1	8	78.91	<0.0001
X_2^2	1	8	1653.44	<0.0001
X_3^2	1	2.67	1923.6	<0.0001
X_4^2	1	2.67	1773.47	<0.0001
$X_1X_2X_3$	1	2.67	19.32	0.0023
$X_1X_2X_4$	1	8	2.29	0.169
$X_1X_3X_4$	1	8	2.25	0.1723
$X_2X_3X_4$	1	8	3.59	0.0946
$X_1^2X_2$	1	8	99.02	<0.0001
$X_1^2X_3$	1	8	141.07	<0.0001
$X_1^2X_4$	1	8	28.32	0.0007
$X_1X_2X_3X_4$	1	8	3.32	<0.0001

[a] X_1: concentration of urea; X_2 – inoculum level; X_3 – pH; X_4 – time.

3.4 Optimization of calcium carbonate precipitation by *Klebsiella pneumoniae*

Central composite rotatable design (CCRD) was employed to determine the correlation between the independent variables and the response (Table 4).

$$\begin{aligned} Y = {} & +9.98 + 0.073X_1 - 0.024X_2 - 0.024X_3 + 0.025X_4 \\ & + 0.017X_1X_2 + 1.71X_1X_3 + 6.515X_1X_4 \\ & - 4.935X_2X_3 - 7.460X_2X_4 - 0.014X_3X_4 \\ & - 0.064X_1^2 - 0.073X_2^2 - 0.078X_3^2 - 0.075X_4^2 \\ & - 7.121X_1X_2X_3 - 2.449X_1X_2X_4 - 2.428X_1X_3X_4 \\ & + 3.071X_2X_3X_4 + 0.028X_1^2X_2 + 0.033X_1^2X_3 \\ & - 0.015X_1^2X_4 - 0.15X_1X_2^2 + 2.51X_1X_2X_3X_4 \end{aligned}$$

where Y is the predicted response for precipitated calcium carbonate and X_1, X_2, X_3 and X_4 are urea concentration, inoculum level, pH and time, respectively.

The analysis of variance (ANOVA) of suggested quadratic regression model demonstrated that the model was significant as indicated by F-value with a very low probability (Table 4). The R^2 value of 1 for response suggested a very less difference or negligible difference between the calculated and observed value and thus indicating the well fitness of regression models for predicting the precipitation results. All the p-values were found to be less than 0.05 which indicated that these variables had a significant effect upon the calcium carbonate precipitation.

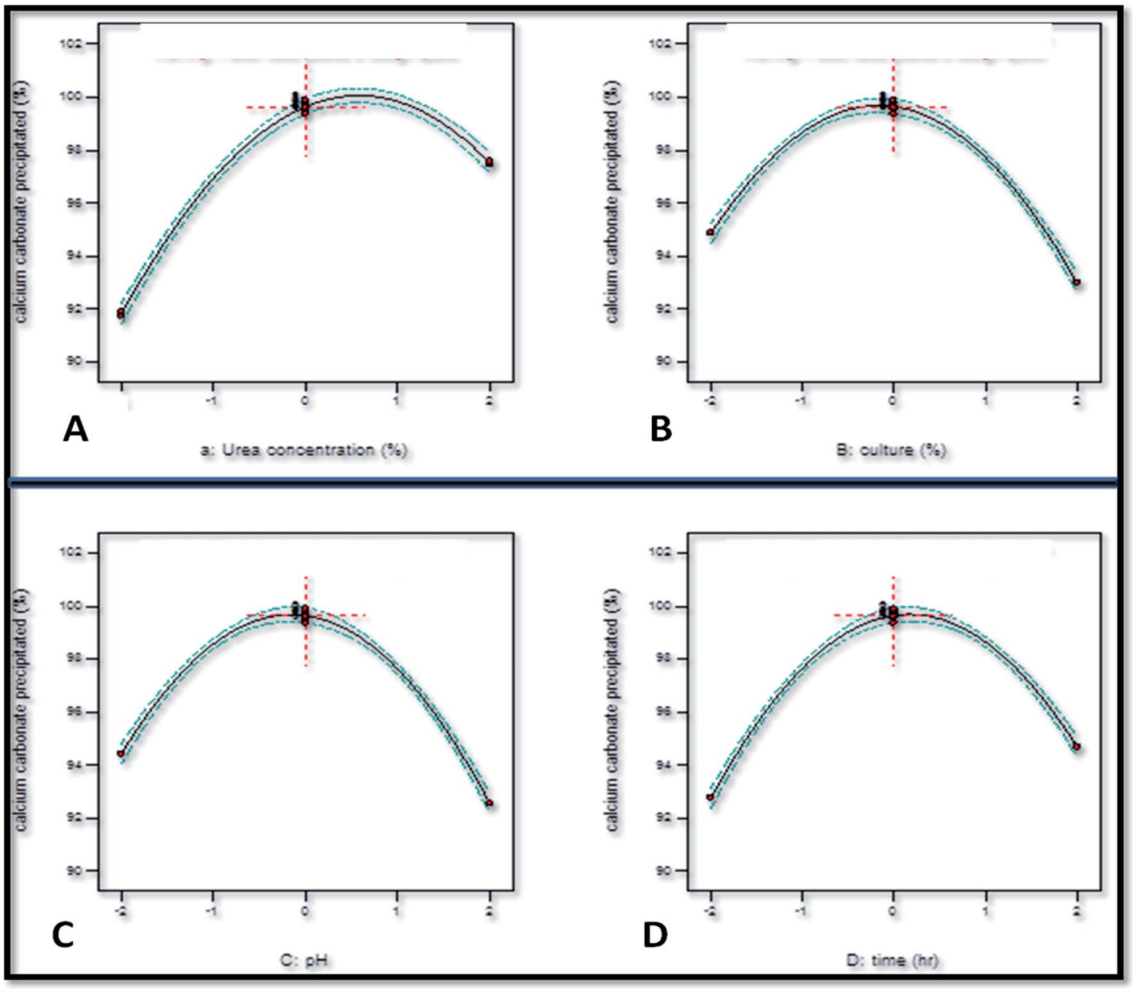

Fig. 4 Linear effect of independent variables upon calcium carbonate precipitation.

This implied that the linear as well as quadratic effects of all four factors were highly significant ($p < 0.0001$) at the 5% significance level.

The individual effect of increasing the concentration of urea was an augmentation in the calcium carbonate precipitation as the coefficient value for X_1 obtained was +0.073 (Fig. 4). However, increasing the initial concentration of urea beyond a limit exerted a negative effect on calcium carbonate precipitation as the coefficient of ${X_1}^2$ obtained was −0.064. This observation corroborated well with the study of Okyay and Rodrigues[33] who reported the positive effect of urea concentration on calcium carbonate precipitation by *Sporosarcina pasteurii* using response surface methodology as the value of X_1 = +0.106. However, beyond a limit, the increase in the initial concentration of urea exerted a negative effect on calcium carbonate precipitation as the value of ${X_1}^2 = -0.016$. The linear coefficient value of −0.024 as well squared coefficient value of −0.073 of inoculum level indicated that the rate of calcium carbonate precipitation decreased with the increase in the inoculum level. pH was also found to have a negative effect on calcium carbonate precipitation as the linear coefficient value obtained was $X_3 = -0.024$. Time was found to have a positive effect as the coefficient value obtained was +0.025.

The interactive effect of factors on calcium carbonate precipitation was analyzed by the construction of three-dimensional (3-d) graphical response (Fig. 5) in which two factors were varied while keeping third factor constant. The interaction of urea concentration and inoculum level was found to exert a positive effect upon the calcium carbonate precipitation as the coefficient value of this interaction obtained was $X_1X_2 = +0.017$. The interaction of urea concentration and pH was also found to have a positive influence upon calcium carbonate precipitation ($X_1X_3 = +1.71$). The most significant positive effect was found to be of interaction of urea concentration and time ($X_1X_4 = +6.515$). The interaction of inoculum level and pH was found to exert a negative effect ($X_2X_3 = -4.935$). The study of interaction between the variables can be extrapolated for understanding the effect of variables on calcium carbonate precipitation and thus formation of speleothems in caves. Similar observations have been reported by Li *et al.*[31] and Daskalakis *et al.*[17] who studied calcium carbonate precipitation ability of a mutant strain of *Sporosarcina pasteurii* and *Cupriavidus metallidurans*, respectively.

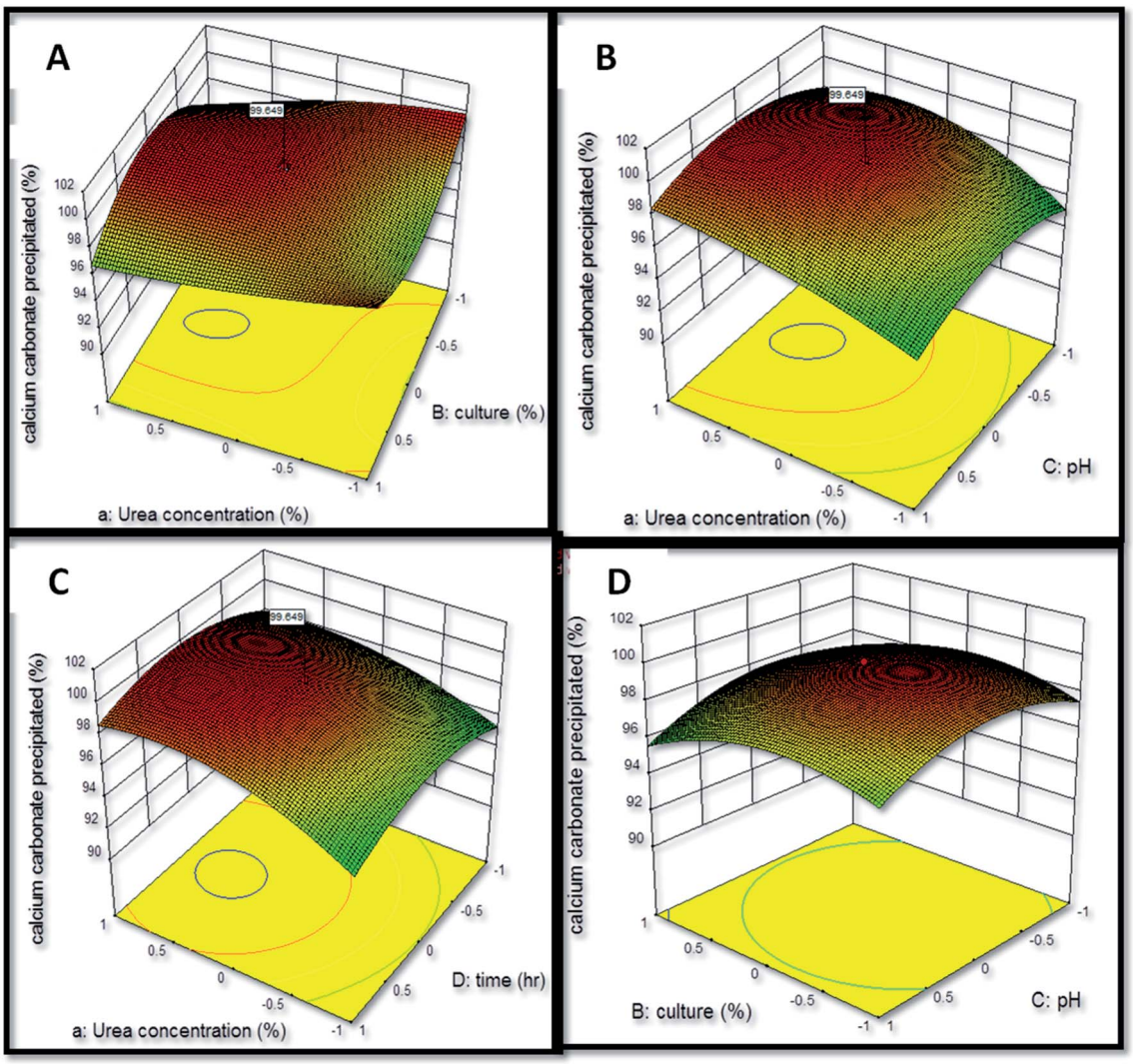

Fig. 5 Effect of interaction of independent variables on calcium carbonate precipitation.

Table 5 Amount (%) of calcium carbonate precipitated by *Klebsiella pneumoniae* before and after optimization[a]

	Optimization condition			% of calcium carbonate precipitated		
Variable	Before	Predicted	Actual	Before	Predicted	Actual
Urea concentration (%)	1.00	1.50	1.5625	97 ± 0.23	99.614	99.879 ± 0.04
Inoculum level (%)	10.00	20.00	19.98			
pH	6.00	7.00	6.98			
Time (h)	60 h	36 h	38 h 24 min			

[a] Value represent mean ± SD.

3.4.1. Validation of model. The validation experiments with RSM suggested optimized conditions for calcium carbonate precipitation were: 1.5% urea concentration, 20% inoculum level, pH 7.0 and 36 h (Table 5) and the predicted amount of calcium carbonate precipitated was 99.614%. The actual amount of calcium carbonate obtained after validation experiment was 99.879 ± 0.04 at 1.5625% urea concentration, 19.98% inoculum level, pH 6.98 and 38 h 24 min time. An increase of 2.96% in amount of calcium carbonate precipitated was obtained after process optimization. A significant finding of present study is that *Klebsiella pneumoniae* precipitated all calcium present in the medium in 38 h 24 min while several species of *Bacillus* had been reported to precipitate calcium in 9–20 days.[6] No cave dwelling bacteria has been reported to precipitate calcium so rapidly.

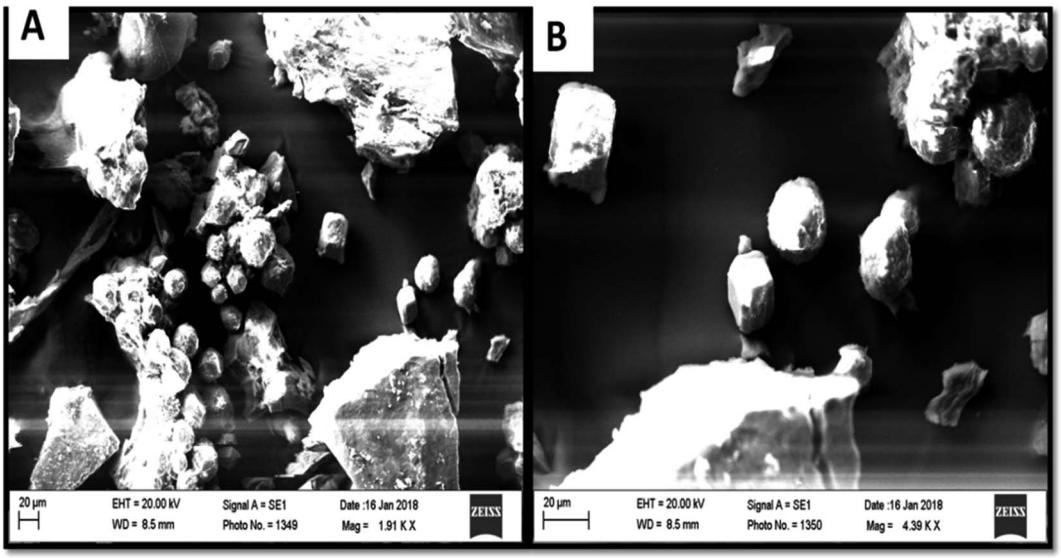

Fig. 6 Scanning electron images of precipitated calcium carbonate crystals.

Table 6 Elements present in precipitated calcium carbonate crystals

Elements	Weight (%)
C	50.97
O	37.14
Na	1.41
P	0.92
Cl	0.96
K	0.36
Ca	8.24

3.5 Analysis of precipitated calcium carbonate crystals

The precipitation of calcium carbonate crystal (Fig. S4†) was proved by SEM-EDX and XRD analysis. The round and cubical shaped particle clearly visible in the SEM image indicated the production of the crystal (Fig. 6). EDX analysis revealed that the major elements in the crystal were carbon (50.97%), oxygen (37.14%) and calcium (8.24%) (Table 6 and Fig. 7). XRD analysis confirmed the crystalline structure and revealed the size of precipitated sample (Fig. 8). The prominent peak matched with the ICSD reference code 01-072-4582 calcium carbonate with 2θ value = 29.938. The displacement value (2θ value) of crystal was obtained 0.397 and the size of crystal size was found to be 166 Å. The shape of crystal shape was found to be rhombohedral.

4. Conclusions

The present study confirmed the precipitation of calcium carbonate by bacteria isolated from stalactite of Sahastradhara cave indicating the biogenic origin of speleothems in the cave. *Klebsiella pneumoniae* was able to precipitate calcium carbonate in 38 h 24 minute which is the shortest time reported till date by cave microbiota. Calcium precipitating ability of cave

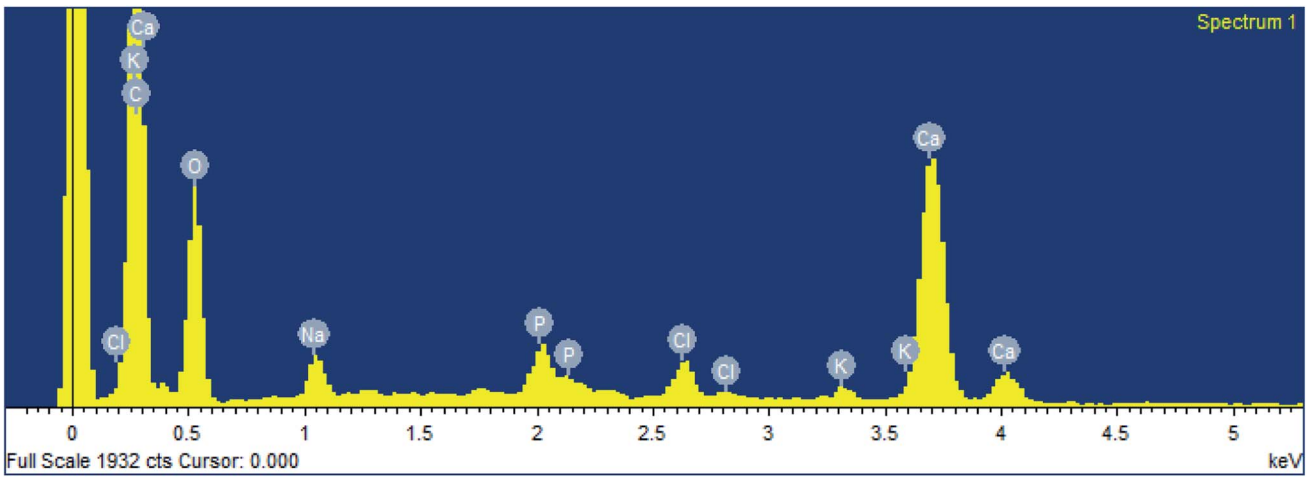

Fig. 7 EDX analysis of precipitated calcium carbonate crystals.

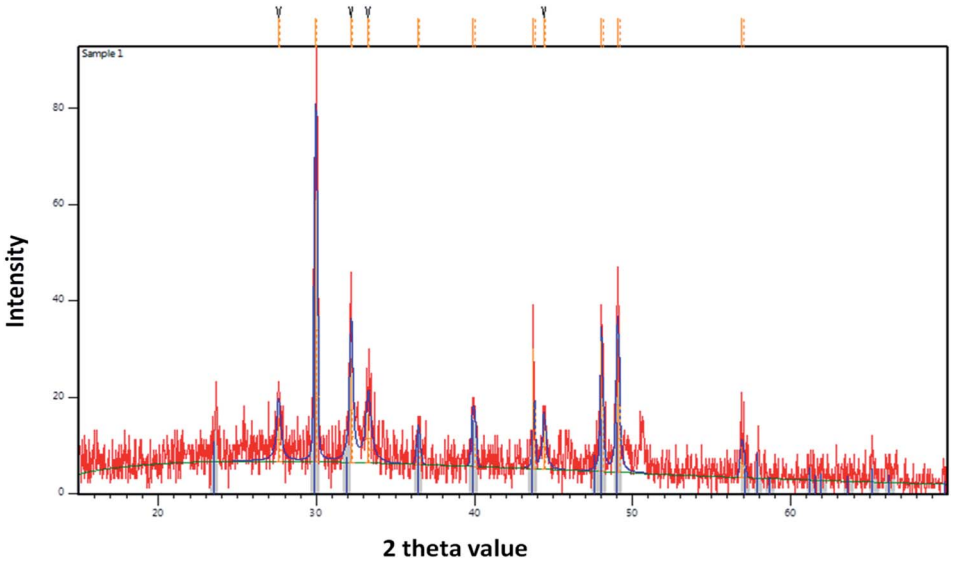

Fig. 8 XRD analysis of precipitated calcium carbonate crystals.

microorganisms can be exploited in construction industries as the microorganisms can repair the cracks in concrete by producing calcite crystals. *Klebsiella pneumoniae* can serve as potential bacterium for formation of bioconcrete.

Conflicts of interest

There are no conflicts of interest.

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