J Appl Biotechnol Rep. 2021 September;8(3):303-311

Journal of
Applied Biotechnology Reports

doi 10.30491/JABR.2020.245335.1278

Original Article

Process Optimization of Microbially Induced Calcite Precipitation by Ureolytic Yeast *Spathospora* sp. NN04 using Box-Behnken Design: A Novel Approach towards Biocementation

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Received August 25, 2020; **Accepted** October 20, 2020; **Online Published** September 25, 2021

Abstract

Introduction: The present study was focused on the statistical optimization of growth parameters for enhancing the Microbially Induced Calcite Precipitation (MICP) using ureolytic yeast strain.

Materials and Methods: Thirteen yeast strains were tested for the synthesis of urease enzyme by phenol-hypochlorite assay and were further evaluated for calcite precipitation test. The growth parameters were optimized using the best ureolytic strain by Box-Behnken Design (BBD) and the extracted MICP was characterized through instrumental analysis.

Results: Among thirteen yeast strains, *Candida tropicalis* NN4, *Spathospora* sp. NN04, *Wickerhamomyces anomalus* VIT-NN01 and *Candida dubliniensis* NN03 showed positive results for the synthesis of urease enzyme. *Spathospora* sp. was found to be the most potent strain for MICP. A significant enhancement in MICP by *Spathospora* sp. was observed under optimized conditions viz. A-urea concentration (80.0 g/L), B-calcium chloride (45.0 g/L), C-pH (9.0) and D-inoculum dosage (8%, v/v). The actual value (34.4±0.12 g/L) was in agreement with predicted value (34.7±0.01g/L) with the R^2 value (0.9900), confirming the validity of the model. The FTIR of MICP confirmed the fundamental bands of CO_3 stretching and bending vibrations, observed at 1394.23 and 874.85 cm^{-1}. The Scanning Electron Microscope (SEM) images of biomotar revealed aggregated polymorphs of MICP interconnected with yeast mycelium and spores. The Energy Dispersive X-Ray Spectrometer (EDX) analysis indicated the presence of calcite in the biomotar. A remarkable improvement in the compressive strength (28 to 44 MPa) and morphological changes were observed in biocement mortar as compared to cement mortar.

Conclusions: This result is the first report on the implementation of ureolytic *Spathospora* towards the application of biocementation through MICP using BBD.

Keywords: Biocementation, Box Behnken Design, Compressive Strength, Microbially Induced Calcite Precipitation, Ureolytic Yeast *Spathospora* sp. NN04

Citation: Ojha N, Aich P, Das N. Process Optimization of Microbially Induced Calcite Precipitation by Ureolytic Yeast *Spathospora* sp. NN04 using Box-Behnken Design: A Novel Approach towards Biocementation. J Appl Biotechnol Rep. 2021;8(3):303-311. doi: 10.30491/JABR.2020.245335.1278

Introduction

Microbially induced calcite precipitation (MICP) is considered as a biologically supported calcium carbonate ($CaCO_3$) precipitation technology. Two mechanisms such as biologically controlled and biologically induced $CaCO_3$ precipitation are involved in this technology. In case of biologically controlled mechanism, microorganisms control the growth and nucleation of the mineral particles. The minerals are synthesized in a form which is unique to the microbial species irrespective of the environmental conditions.[1] However, in the case of biologically induced $CaCO_3$ precipitation mechanism, the production of $CaCO_3$ precipitation is found to be solely dependent on environmental conditions.[1,2]

Many researchers have reported MICP process driven by ureolysis which has been used widely for remediation of concrete cracks, reduction of soil erosion, stabilization and strengthening of soil, restoration of limestone surfaces, treatment of different types of wastewater and removal of toxic heavy metals.[3-8] The efficacy of ureolysis driven MICP process has also been reported in the field of biosensor, agriculture and healthcare application.[9] In addition, MICP is frequently used as a construction and building material for the production of $CaCO_3$ as a bio-calcifying agent to improve the geotechnical properties.

The application of microorganism as an effective catalyst in case of soil biocementation has drawn much attention, which relies on the process of MICP involving urease producing microorganism, mainly bacteria.[9-12] *Bacillus pasteurii* or *Sporosarcina pasteurii* has been reported as the most suitable bacteria in the literature for the MICP process. The bacteria are capable of producing a high amount of

calcium carbonate precipitates within a short period of time because of high urease producing capacity.[13-15] The microbial urease catalyzed biocementation process has been widely used for the preparation of construction materials, geotechnical engineering and environmental applications[16-17] and for the remediation of damaged structural formations.[18-19] This process follows the steps of complex biochemical reactions involving the participation of urease producing alkalophilic soil bacteria at high pH.[13] The hydrolysis of urea to ammonia and carbon dioxide occurs due to the urease enzyme produced by bacteria. The pH of the surroundings increases due to ammonia which induces calcium carbonate precipitation.[20]

Although much success has been achieved for this technology at laboratory-scale, MICP is still not approved for commercial implementation or field-scale applications as the process is not cost effective.[21] The cost of nutrient medium used in the biotechnological process for the cultivation of bacteria may be one of the reasons.[22] The major cost for bacterial cultivation involves laboratory-grade growth media which may reach up to 60% of the total production cost.[23]

Concrete is a widely used construction material,[24] but it cracks easily due to its low tensile strength. Fast crack healing is needed as it is easy for aggressive substances to enter into the concrete via cracks compared to concrete matrix. It is well known that the process including inspection, monitoring and repairing of cracks is quite expensive. Therefore, it is desirable to heal the concrete cracks through calcite precipitation. There are reports of calcite precipitation induced by ureolytic bacteria viz. *Bacillus pasteurii*,[13] *Sporosarcina pasteurii*,[25] *Bacillus sphaericus*,[24] *Bacillus sp.* CT-5,[26] *Lysinibacillus sphaericus*,[27] *Bacillus pseudofirmus*[28] and *Acinetobacter* sp. SC4,[29] *S. pasteurii* ATCC 11859,[30] and *Lysinibacillus sphaericus*.[31] It has been also noted that MICP could be applied for protecting the limestones using the techniques of surface treatments [32].

So far, no report is available on ureolytic yeasts showing the capacity of calcite precipitation which is needed for healing the cracks. Response Surface Methodology (RSM), a powerful statistical tool, has been applied for the prediction of the optimization of growth parameters in many analytical fields. However, reports are scanty on statistical optimization of growth parameters for induced calcium carbonate precipitation using bacteria.[33]

The aim of this study is a screening of urease producing yeast strains showing the capacity of calcite precipitation, optimization of induced calcium carbonate precipitation using RSM, characterization of calcite and to improve the compressive strength of cement mortars.

Materials and Methods

Microorganisms

The thirteen yeast strains viz. *Yarrowia lypolytica* VIT-MN01, *Kluveromyces lactis* VIT-MN02, *Lipomycis starkeyi* VIT-MN03, *Sacchromycopsis fibuligera* VIT-MN04, *Brettanomyces custersianus* VIT-MN05, *Rhodotorula* sp. NS01, *Hansaniaspora opuntiae* NS02, *Debaryomyces hansenii* NS03, *Hanseniaspora valbyensis* NS04, *Candida tropicalis* NN4, *Spathospora* sp. NN04, *Wicherhamomyces anomalus* VIT-NN01 *and Candida dubliniensis* NN03 were collected from the bioremediation laboratory, Vellore Institute of Technology, Vellore, Tamil Nadu and sub-cultured on yeast extract peptone dextrose (YEPD) agar medium.

Screening of Urease Producing Yeast Strains

The urease enzyme produced by microorganisms play an important role in the precipitation of calcite.[20] This hypothesis leads to the screening of urease producing yeast strains by phenol-hypochlorite assay method using Christensen's medium (Himedia, India) containing the following composition (g/L) of peptone (1.0), dextrose (1.0), sodium chloride (5.0), monopotassium phosphate (2.0), phenol red (0.012), agar (15.0) and 40% sterile urea solution which were incubated for 48 h at 30 °C.

Synthesis of MICP

The capability of the ureolytic yeast isolates to precipitate calcite were selected by culturing the strains in the calcite precipitation medium (pH 6.5) which contained (g/L) nutrient broth (3.0), $CaCl_2$ (28.5), $NaHCO_3$ (2.12) and urea (20.0)[34] and were incubated for four days at 30 °C. The precipitated $CaCO_3$ crystals were filtered with Whatman filter paper and dried for 48 h in oven at 60 °C. The dried weight of precipitated $CaCO_3$ crystals induced from ureolytic yeast strains was estimated gravimatically.[35]

Characterization of Synthesized MICP

Instrumental analysis was done in order to characterize the calcite precipitated by the ureolytic yeast *Spathospora* sp. NN04. The dried crystals of Induced Calcite Precipitate (ICP) was powdered into fine particles and subjected to elemental analysis through UV visible spectrophotometer and Fourier Transform iInfrared (FTIR) spectroscopy analysis following the standard method.[36] Morphological characterization and elemental mapping of ICP were done using SEM equipped with the EDX analysis. The carbon coated samples at an accelerating voltage of 20 kV were used for EDX qualitative analysis and elemental composition.[37]

Statistical Optimization of Growth Parameters

The BBD was used to enhance the calcite precipitation by ureolytic yeast *Spathospora* sp. NN04 varying the parameters. A set of 29 trials were experimented to determine the influence and interaction of the four variables viz. urea concentration (mg/L), sodium chloride (mg/L), pH and inoculum dosage (%, v/v), each variable with three

distinct levels of low (-1), medium (0) and high (+1) on response as dry weight of calcite precipitation (g L^{-1}) as tabulated in Table 1. The statistical evaluation of the design was executed to examine the analysis of variance (ANOVA). All the experimental trials and regression evaluation were done by the Design Expert software (Version 11). The potentiality of polynomial model equation was concluded by elucidating the coefficient R and its statistical importance was recognised by F-test. All experimental trials were executed in triplicate. The achieved results were emphasized as the average of three biological replicates ± standard deviation (SD).

Table 1. Independent Factors and Its Level Used in Response Surface Design for Dry Weight of Calcite Precipitation

Factors	Name	Low level (-1)	Level (0)	High level (+1)
A	Urea concentration (g/L)	50	80	110
B	Calcium chloride (g/L)	5	45	85
C	pH	5	9	13
D	Inoculum dosage (%, v/v)	4	8	12

Compressive Strength of the Biocement Mortar

Based on the RSM results, the optimal concentration of calcium chloride and urea were used as essential components which were important in the growth of yeast and induction of calcite precipitation. The culture medium was totally suspended in distilled water heated at 80 °C followed by cooling at room temperature before mixing. Mortar was prepared by mixing Hydraulic cement (KSL 5201) and standard sand (KSL ISO 679) at 3:1 ratio with distilled water. Four distinct test samples were formulated, viz., standard mortar sample (Sand + Cement; SCe) as a control, mortar test sample with yeast cells (Sand + Cement + Yeast cells; SCeY), mortar test sample mixed with culture medium (Sand + Cement + Culture medium; SCeCu) and mortar test sample mixed with culture medium and yeast cells (Sand + Cement + Culture medium + Yeast cells; SCeCuY). The compressive strength test of all the four samples were evaluated to observe the effect and interaction of ICP on the strength of cement mortar. The compressive strength of the specimens was measured by the KSL 5105 testing method,[27] in which the samples were treated till days seven and 28 in an environmental chamber bearing a temperature of 20 °C and a relative humidity of 90%. The compressive strength test of the specimens was executed in triplicates (n = 3), and the results were estimated in mean value with standard deviation (Mean ± SD). All the statistical analysis was done using Graph Pad Prism software (Version 5.03).

Surface Topology and Elemental Mapping of the Biocement Mortar

Morphological and surface topological observations of the cement mortar and yeast mediated biocement mortar were carried out using the SEM (Edwards S150B) analysis.[38] The elemental distribution/mapping of the cement mortar and yeast mediated biocement mortar were performed using EDX analysis on the carbon-coated samples at the accelerating voltage of 200 V to 30 kV.[37]

Table 2. ANOVA for Response Surface Quadratic Model (Response: Dry Weight of Calcite Precipitation g/L)

Source	Sum of Squares	Degree of freedom	Mean Square	F-value	*P*-value	
Model	26.55	14	1.90	98.57	< 0.0001	Significant
A-Urea concentration	0.1584	1	0.1584	8.23	0.0124	Significant
B-Calcium chloride	0.0043	1	0.0043	0.2243	0.6431	Significant
C-pH	0.0524	1	0.0524	2.72	0.1213	Significant
D-Inoculum dosage	22.17	1	22.17	1151.96	< 0.0001	Significant
AB	0.2525	1	0.2525	13.12	0.0028	Significant
AC	0.0046	1	0.0046	0.2368	0.6341	
AD	0.2426	1	0.2426	12.61	0.0032	Significant
BC	0.0028	1	0.0028	0.1432	0.7108	
BD	0.0116	1	0.0116	0.6006	0.4512	
CD	0.0189	1	0.0189	0.9826	0.3384	
A	0.0729	1	0.0729	3.79	0.0520	Significant
B	0.0009	1	0.0009	0.0488	0.8283	
C	0.0006	1	0.0006	0.0291	0.8671	
D	0.0051	1	0.0051	0.2637	0.6156	
Residual	0.2694	14	0.0192			
Lack of Fit	0.2261	9	0.0251	2.90	0.1266	Not Significant
Pure Error	0.0433	5	0.0087			
Cor Total	27.33	28				
Std. Dev.	0.1387					
Mean	3.52					
C.V. %	3.94					
R	0.9900					
Adjusted R	0.9799					
Predicted R	0.9369					
Adeq. Precision	37.4267					

Table 3. Actual Versus Predicted Value for Response: Dry Weight of Calcite Precipitation (g/L)

Std	Run	Factor 1	Factor 2	Factor 3	Factor 4	Response :Dry weight of Calcite (g/L)	
		A: Urea concentration	B: Calcium chloride	C: pH	D: Inoculum dosage	Actual value	Predicted value
16	1	+1	+1	+1	+1	35.7	35.6
29	2	0	0	0	0	34.5	34.7
1	3	-1	-1	-1	-1	21.8	23.8
21	4	0	0	0	0	33.2	34.7
14	5	+1	-1	+1	+1	34.3	35.3
18	6	+1	0	9	0	35.3	35.7
9	7	-1	-1	-1	+1	34.9	34.8
10	8	+1	-1	-1	+1	35.2	36.8
15	9	-1	+1	+1	+1	34.5	33.6
22	10	0	0	+1	0	35.4	34.5
13	11	-1	-1	+1	+1	33.5	34.3
2	12	+1	-1	-1	-1	26.7	25.8
7	13	-1	+1	+1	-1	21.7	22.6
4	14	+1	+1	-1	-1	26.6	25.1
26	15	0	0	0	0	35.9	34.7
8	16	+1	+1	+1	-1	22.0	24.6
11	17	-1	+1	-1	+1	34.5	34.1
6	18	+1	-1	+1	-1	27.8	25.3
19	19	0	-1	0	0	34.6	34.8
5	20	-1	-1	+1	-1	23.6	23.4
17	21	-1	0	0	0	35.2	32.7
25	22	0	0	0	0	34.7	34.7
3	23	-1	+1	-1	-1	20.2	23.1
23	24	0	0	0	-1	24.5	24.2
27	25	0	0	0	0	35.3	34.7
20	26	0	+1	0	0	34.3	34.0
12	27	+1	+1	-1	+1	35.9	35.0
28	28	0	0	0	0	32.6	34.7
24	29	0	0	0	+1	31.7	35.7

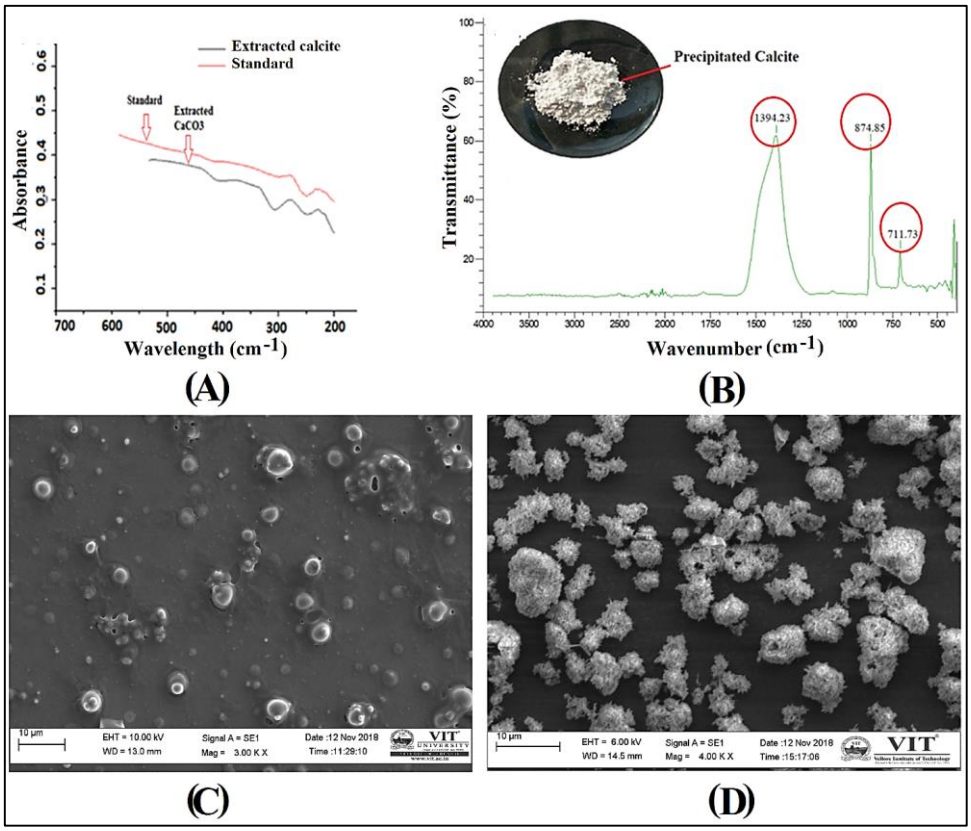

Figure 1. Characterization of Calcite Induced by Ureolytic Yeast *Spathospora* sp. NN04 using (A) UV Vis spectroscopy analysis, (B) FTIR analysis. SEM analysis of (C) Ureolytic yeast *Spathospora* sp. NN04, (D) Precipitated calcite.

Results and Discussion

Screening of Urease Producing Yeast Strains

Among 13 yeast strains, four yeast strains viz. *Candida tropicalis* NN4, *Spathospora sp.* NN04, *Wickerhamomyces anomalus* VIT-NN01 and *Candida dubliniensis* showed the capability to produce urease enzyme (Supplementary Figure S1) and were further evaluated for the calcite precipitation test. The other nine yeast strains did not show the capabilities to produce urease enzyme resulting no colour change in the medium (Supplementary Figure S1).

Synthesis and Characterization of MICP

The maximum calcite precipitation was shown by *Spathospora* sp. NN04 and the dry weight of the total calcite precipitation was found to be 6.5 g/L. Analytical tests were carried out in order to characterize the calcite precipitated by the ureolytic yeast *Spathospora sp.* NN04. The UV visible spectrum (Figure 1A) of the standard calcite and yeast induced calcite were corresponding to each other which confirmed the precipitation of calcium carbonate. The FTIR spectrum represented in Figure 1B showed CO_3 stretching and bending vibration bands at 1394.23 and 874.85 cm^{-1} respectively, which were corresponding to the fundamental bands for calcite.[36] The SEM micrograph shown in Figure 1C indicated the spherical shape of the yeast cells. The surface topological studies of the precipitated calcite revealed agglomeration, polymorphs and needlelike microstructures by SEM analysis (Figure 1D).

Statistical Optimization of Growth Parameters

The ANOVA for the quadratic model of response has been presented in Table 2. Based on the result, the maximum dry weight of calcite precipitation was found to be 34.3 ± 0.12 g/L at central values of all the variables viz., Urea concentration (80.0 g/L), Calcium chloride (45.0 g/L), pH (9) and Inoculum dosage (8%, v/v) using ureolytic yeast *Spathospora* sp. NN04 after an incubation period of six days. The R^2 (0.9900), adjusted R^2 (0.9799), predicted R^2 (0.9369), F-value (144.70), coefficient of variation (3.94 %) and probability of <0.0001 confirmed that the model is highly significant and the experiments are accurate and reliable. An adequate precision (37.4267) for the response also supports the model. The lack of fit F-value (2.99) is not significant compared to the pure error as presented in Table 2. Based on the statistical importance, the second order polynomial equation for the response can be written as:

$$Y = +34.4 + 0.0729\,A - 0.0308\,B + 0.0399\,C + 1.10\,D + 0.1131\,AB + 0.0044\,AC - 0.1106\,AD - 0.0256\,BC + 0.0394\,BD - 0.0219\,CD + 0.0588\,A^2 + 0.0244\,B^2 + 0.1026\,C^2 - 0.0909\,D^2$$

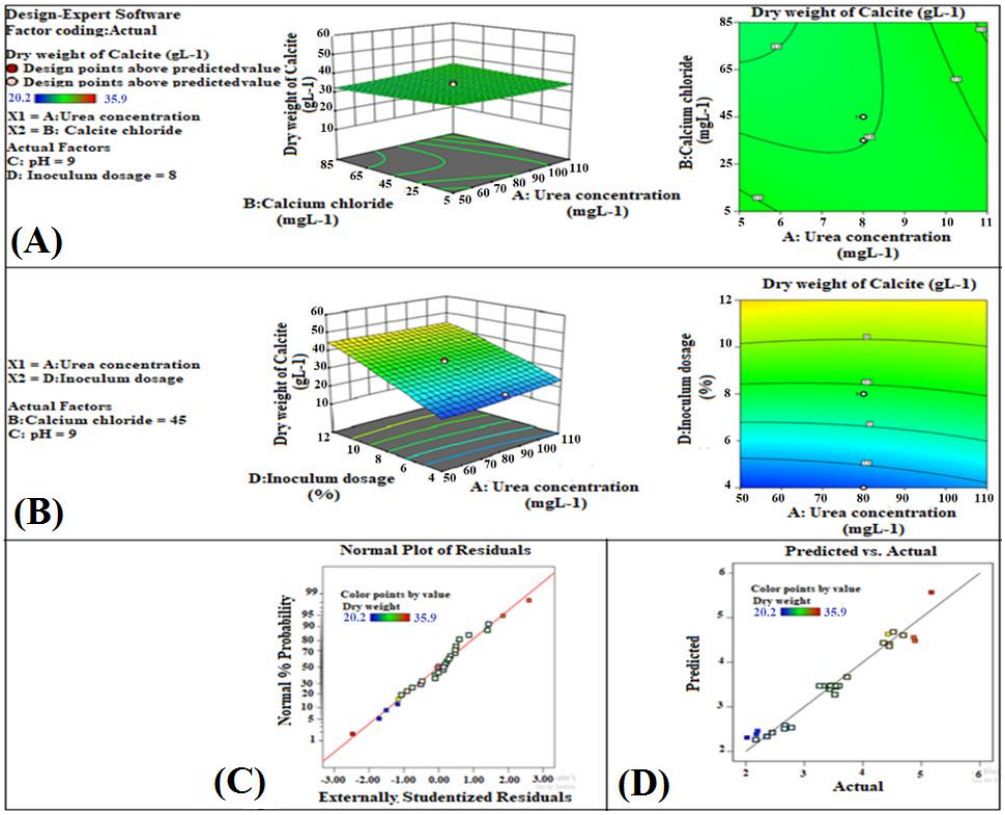

Figure 2. 3-D and Contour Interactions between the Different Variables for Response: Dry Weight of Calcite Precipitation (g/L). (A) Urea concentration vs Calcium chloride (AB), (B) Urea concentration vs Inoculum dosage (AD), (C) Normal plot of residuals, (D) Predicted vs actual values.

Table 4. ANOVA Results for Compressive Strength of Biocement Motars

Source of Variation	Df	Sum-of-Squares	Mean Square	F-value	% of Total Variation	*P*-value	
Interaction	3	15.12	5.041	5.568	1.14	0.0082	**
Column Factor	1	209.2	209.2	231.1	15.79	< 0.0001	***
Row Factor	3	1086	362.1	400.0	81.98	< 0.0001	***
Residual	16	14.48	0.9053				

*** Significant; *** Highly Significant*

Table 5. Elemental Mapping of Cement Mortar by EDX Analysis

Elements	Cement Mortar (Control)		Biocement Mortar (Test Sample)	
	Weight (%)	Atomic (%)	Weight (%)	Atomic (%)
Carbon (C)	00.00	00.00	40.34	54.09
Oxygen (O)	43.11	29.85	36.29	36.53
Silica (Si)	54.49	66.25	13.00	07.37
Calcium (Ca)	02.41	03.90	10.37	02.01
Totals	100.00		100.00	

Where Y represents the dry weight of calcite precipitation (g/L) as a response and A, B, C and D were coded terms for the four test factors viz. Urea concentration (g/L), Calcium chloride (g/L), pH and Inoculum dosage (%, v/v) respectively. Among all, A, D, AB, AD and A^2 were found to be significant model terms ($P < 0.05$) (Table 2). The interaction between the variables urea concentration vs calcium chloride (AB) as well as urea concentration vs inoculum dosage (AD) had shown maximum calcite precipitation (34.4 g/L) as shown in Figure 2A and Figure 2B respectively.

The statistical model was confirmed by implementing point prediction tool of RSM from an optimal value of all four factors A, B, C, and D. Figure 2C and Figure 2D represent the normal plot for residuals and predicted vs actual plots respectively. The actual dry weight of calcite precipitation (34.4 ± 0.12 g/L) was in close agreement with the predicted value (34.7 ± 0.01 g/L) demonstrating the rationality of the model (Table 3). Thus, a remarkable enhancement from 6.5 to 34.4 g/L in the dry weight of calcite precipitation was observed by ureolytic yeast *Spathospora* sp. NN04 in the aqueous medium under optimized condition. Reports says that 33.78 g/L of calcite precipitation was induced using *Bacillus* sp. under optimum conditions.[33] However, the present study has shown the maximum calcite precipitation of 34.4 g/L under optimized conditions using urease producing yeast *Spathospora* sp. NN04.

Compressive Strength of Biocement Mortar

The compressive strength of biocement mortar compiled of mortar with urea concentration (80.0 g/L), calcium chloride (45.0 g/L) and yeast cells (8%, v/v) was found to be most effective as compared to the control (Figure 3). The compressive strength of the mortar mixed with culture medium (SCeCu) was found to be 27.43 MPa (7^{th} days cure) and 34.39 MPa (28^{th} days cure) which was quite higher than control mortar (SCe), as shown in Figure 3. However, the compressive strength of the mortar specimens mixed with yeast cells without a medium (SCeY) was similar to those of the control specimens (20.63 MPa in 7 days & 28 MPa in 28 days). The compressive strength of mortar mixed with *Spathospora* yeast cells and culture medium (SCeCuY) was found to be 40.07 MPa (7 days cure) and 43.37 MPa (28 days cure) which was remarkably better than those of the control specimens showing the novelty of this study. Thus, the use of ureolytic yeast *Spathospora* sp. with the mortar and optimal culture medium was proved to be an active element in binding with the sand matrix to formulate yeast mediated biocement mortar of suitable compressive strength. It can also be implemented for enhancing the compressive strength of other cementitious materials. A similar report was demonstrated, where improvements in the compressive strength were investigated using calcite-forming *Lysinibacillus sphaericus* WJ-8 into concrete pavements.[27]

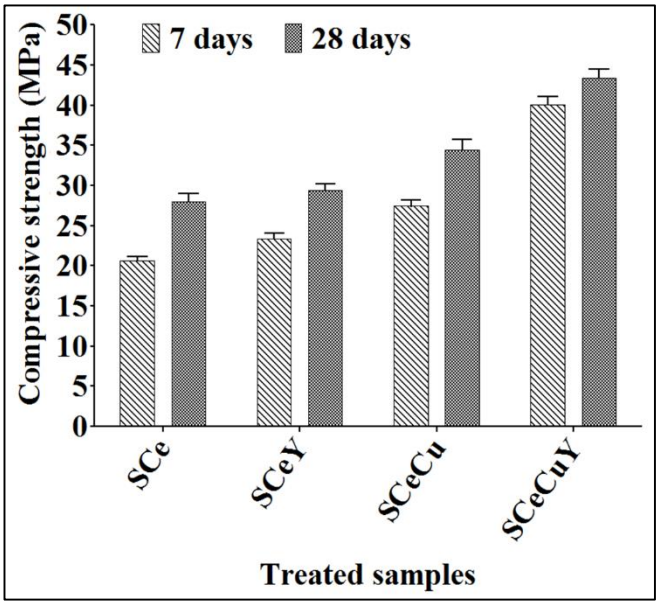

Figure 3. Compressive Strength of the Treated Mortars viz., Sand + Cement (SCe), Sand + Cement + Yeast cells (SCeY), Sand + Cement + Culture medium (SCeCu) and Sand + Cement + Culture medium + Yeast cells (SCeCuY).

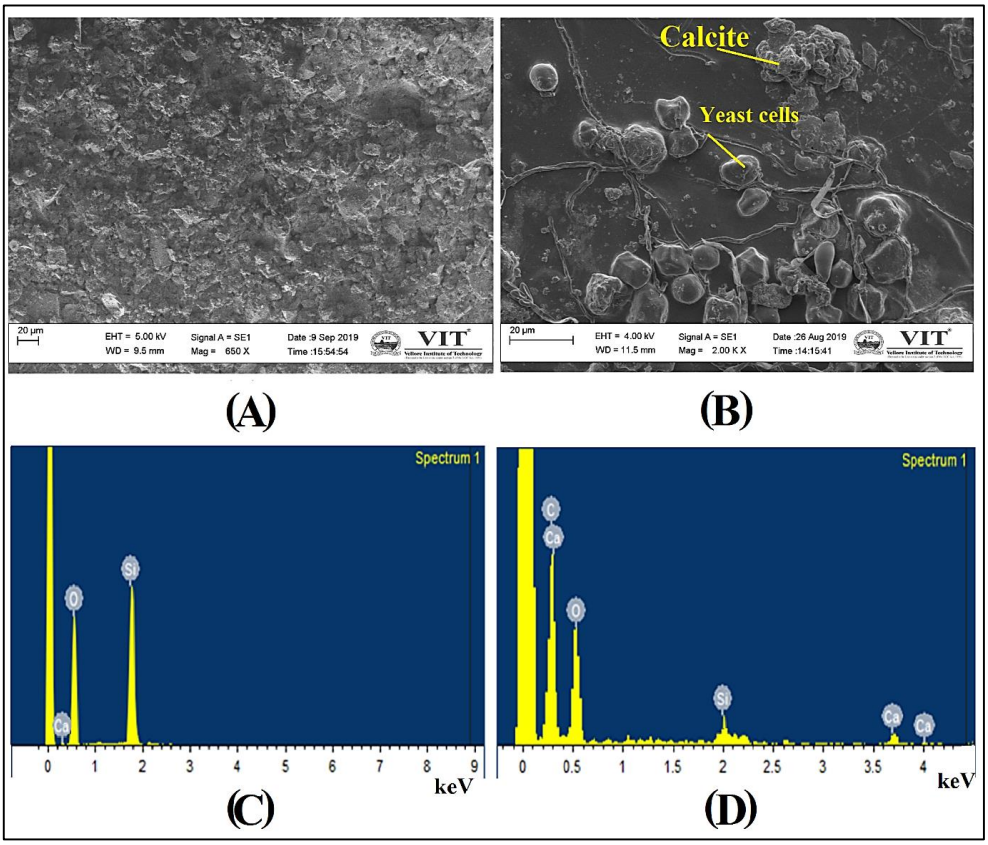

Figure 4. SEM Analysis of (A) Cement mortar (Control, without *Spathospora* sp. NN04), (B) Biocement mortar (treated with *Spathospora* sp. NN04). EDX analysis of (C) Cement mortar (Control, without *Spathospora* sp. NN04), (D) Biocement mortar (treated with *Spathospora* sp. NN04).

All the four different sets of experiments (SCe, SCeY, SCeCu and SCeCuY) were executed in triplicates and the findings were examined by two-way ANOVA (Table 4). The ANOVA results showed that the *P*-value <0.0001, specified that the interaction was highly noteworthy.

Surface Topology and Elemental Mapping of the Biocement Mortar

The rough and crack surface topology was observed in the SEM micrograph of cement mortar and the calcite phase was not observed in control cement mortar specimens which were made without any addition of yeast cells as shown in Figure 4A. The SEM micrograph of yeast mediated biocement mortar revealed the excessive growth of ureolytic *Spathospora* sp. cells without any formation of cracks and roughness on the surface and the precipitated calcite by *Spathospora* yeast cells could be clearly distinguished within the pores of the biocement matrix (Figure 4B). Therefore, the biocementation is mainly reliant on the capability of microorganisms to grow easily either by insertion throughout the pore openings or by appropriate particle-particle contacts.[27]

The EDX spectrum showed the elemental composition of the microlayer of cement mortar which consists of only silica, oxygen and very less weight % of calcium as its principal constituent (Figure 4C). However, yeast mediated biocement mortar revealed the presence of 40.34 weight % of carbon, 36.29 weight % of oxygen along with 13.00 weight % of silicon and 10.37 weight % calcium as its major constituent which confirmed the existence of yeast biomass in the mortar as shown in Figure 4D and Table 5. A similar trend was demonstrated where, the polymorphs and particle size of calcium carbonate precipitated were observed using SEM analysis.[37]

Conclusion

It can be concluded that the ureolytic yeast *Spathospora* sp. NN04 is reported for the first time as a potential source for inducing calcite precipitation. Moreover, RSM served as a useful tool for enhancing MICP which may be helpful towards biocementation. The morphological and compressive strength of the yeast modified mortar was improved due to the deposition of the new calcite material by the yeast activity. The compressive strength increased with the addition of optimal concentration of yeast cells; while no strength improvements were noted in cement mortar (without yeast cells). The amount and agglomeration of the precipitated calcite crystals in cement mortar using 8% yeast cells was effective for healing the cracks. More research is needed on the optimization of the MICP process at macro and micro levels before its direct applications to the field.

Authors' Contributions

Conception and design of the study: ND and NO; Acquisition of data and doing the laboratory phase: NO and PA; Analysis and interpretation of data: NO. ND and NO have contributed to drafting the article and critically revising it for important intellectual content.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

Acknowledgements

The authors are thankful to the School of Advanced Sciences (SAS), the School of Bio-Medical Sciences (SBST) and DST-FIST/VIT SEM of Vellore Institute of Technology, India for providing laboratory facilities while conducting this experiment.

Supplementary Materials

Supplementary Figure S1 is attached with this manuscript.

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