Bio Cementation Process in the Concrete using Bacillus Subtilis: A Statistical Modeling Study

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Abstract. The present study shows the bio-cementation efficacy of Bacillus subtilis incorporating three major parameters namely bacterial concentration, curing time, and nutritional proportion. Batch studies incorporating one variable at a time (OVAT) and central composite design (CCD) along with the response surface methodology (RSM) were undertaken to establish the possible relationship of selected parameters with the mechanical properties (compressive strength, flexural strength and split tensile strength) of cement mortar and bacterial concrete. The investigations performed on the basis of OVAT approach indicated that the extreme level of bacterial concentration, curing time, and nutritional proportion produced the highest improvement in mechanical properties of bacterial concrete producing compressive strength, flexural strength and split tensile strength of 79.5 MPa, 6.22 MPa, and 5.01 MPa respectively. Inline to OVAT approach, analysis of variance (ANOVA) also depicted that the individual as well as some of the possible two factor interactive model terms appeared as significant model terms with p-value was less than 0.05. The response predictive efficacy of the model was ascertained with a high $R^2$ value (0.99) and high adequate precision.

1. Introduction

Concrete is one of the most widely used materials for construction across the globe due to its durability, longevity and compressive strength. However, the structures made up of such materials are prone to the deterioration due to aging and the action of aggressive agents. There are several means which induce the deterioration including alkali-aggregate reactions, abrasion, fire, corrosion of steel bars etc. However, the corrosion of the steel bars can be arrested for an extended period by employing an intact concrete cover [1]. Moreover, the tensile stresses induced in the structures under certain loads may results in the formation of cracks which create a weak zone, acts as an active site of action for aggressive agents. Such cracks grow in number and magnitude during the service life of structures and thereby affect the durability of infrastructure. Consequently, the infrastructure requires repair within a very short period of time and involves a significant repairing and maintenance cost [2]. Maintenance cost increases exponentially due to concrete deterioration because of aging including the increases in the urban population which reside in such structure [3]. Additionally, in 2008, 8% of world CO₂ emissions (greenhouse gas emissions) were produced by cement and construction industry, which was regarded as the most pollutant generation anthropogenic activity [4]. Thus, the aim of improving the
service life of any infrastructure becomes priority considering both economic and environmental prospective [5]. An extended service life generally minimise the requirement of new infrastructure and also diminishes the generation of greenhouse gas emission. On the view of this, the concept of bio-cementation and/or self-healing concrete came into the picture for its possible applicability to improve the sustainability of infrastructure. Here, the structure will regain its strength by auto activation of the crack healing process. In the process of self-healing, the small cracks undergo the repair process automatically under specified environment [6], while bio-cementation generally reduces the resilience of the construction materials by improving the strength.

Figure 1. Comprehensive research taxonomy related to self-healing concrete [7, 8]

Several processes (such as natural, chemical and biological) have been practiced for the design of self-healing concrete as shown in the Figure 1. However, developing self-healing concrete using biological processes has received significant attention among the researchers. In this process, the microorganisms, mainly calcifying bacteria are employed in different forms during the preparation of concrete. As a result, the calcifying bacteria induce the production of CaCO$_3$ (calcite) which occupies the openings present in concrete specimen or fills in the cracks, thereby enhance the mechanical properties by reducing its porosity [3]. Several bacterial strains such as Bacillus, Halomonas, Acinobacter, Pseudomonas, Shewanella, Leuconostoc etc. have been utilized to design the self-healing concrete [9-11]. Out of which Bacillus are the widely used bacterial species. Such studies also reported that ability of different type Bacillus bacteria towards the production of calcite after being stimulated by water.

Several studies suggested that nutritional concentration also affect the calcination process in addition to curing time and bacterial composition. However, there are very limited studies related to the preparation of bacterial concrete and its subsequent statistical assessment with respect to the strength properties. Moreover, none of the studies taken nutritional concentration while statistical modelling. Therefore, in the present study, the statistical modelling was performed for optimization of strength properties (compressive strength, flexural strength and split tensile strength) of the bacterial concrete using Bacillus subtilis as bacterial strain incorporating the effect of three major process parameters namely, bacterial concentration (Cells/mL), curing time (days) and nutritional proportion (%).
2. Material and methods

2.1 Bacterial strain and growth conditions
A rod shaped, gram-positive, ureolytic type bacterium of Bacillus subtilis (CT-5) was chosen since it satisfy the essential criteria of survival in harsh physic-chemical conditions by forming the spores under adverse environment. Autoclaved nutrient broth (Himedia, India) with the standard composition was utilized to cultivate the Bacillus subtilis. Additionally, for in vitro precipitation of calcite, the culture was supplemented with sterilized 2% urea (w/v) and 25 mM CaCl₂ solution at 37°C in the orbital shaker at 120 rpm maintaining a neutral pH. Routine monitoring of growth was done by measuring the absorbance values using UV-Spectrophotometer at 600 nm. Further, a standard calibration curve was prepared using optical density test to assess the volume of culture solution required for different proportions.

2.2 Ordinary Portland cement
An ordinary Portland cement (43 Grade) conforming to BIS 8112-2013 was utilized for the production of concrete. The properties of OPC 43 were determined as per Indian standard specifications (IS 8112:1989) as given in Table 1.

Table 1. Properties of OPC 43

| Physical Properties       | Values         |
|---------------------------|----------------|
| Fineness of Cement        | 2300 cm²/g     |
| Standard Consistency      | 31%            |
| Initial Setting Time (min)| 35             |
| Final Setting Time (min)  | 510            |
| Specific gravity          | 3.25           |

2.3 Fine aggregate and coarse aggregate
Fine aggregate acts as packing material in concrete to fill in the matrix and provide a condensed structure bound by cement. For this purpose, clean, dry and well-graded natural river sand (passing through 4.75 mm sieves) conforming to Zone II was utilized. Physical properties such as specific gravity and water absorption of fine aggregates were measured as 2.70 and 1.8%, respectively. Coarse aggregates provide the strength and resistance to abrasion, which occupies the maximum volume in the concrete. Crushed gravel with downsize of 20 mm was used as the coarse aggregates. The specific gravity and water absorption of aggregates were 2.65 and 1.39%, respectively.

2.4 Statistical Experimental Design
Design Expert software, version 8.0.7.1 was utilized for the experimental design based on the Central composite design (CCD). The interactive effects of three significant factors A (Bacterial concentration), B (Curing time) and C (Nutritional composition) on the response (Mechanical strength properties), were determined statistically using Response Surface Methodology (RSM). These variables A, B and C were considered as zero and investigated at three different levels (Table 2). The value for α (=1) was chosen keeping in mind that extreme factor ranges are less. The factorial points, axial points and the centre points are coded as \((\pm 1, \pm 1, \pm 1), (\pm \alpha, 0, 0), (0, \pm \alpha, 0), (0, 0, \pm \alpha)\) and \((0, 0, 0)\) respectively. A matrix design containing of 12 experiments produced by the software was performed for optimizing the mechanical strength properties (compressive, flexural and split tensile strength).
### Table 2. Original and coded Variable

| Variables                        | Original Variables | Coded Factors |
|----------------------------------|--------------------|---------------|
| Bacterial concentration (×10^3 Cells/ml) | A 5 10 15         | -1 0 +1       |
| Curing time (days)                | B 3 15.5 28       |              |
| Nutritional composition (%)       | C 10 20 30        |              |

#### 2.5 Experimental Design and Data Analysis for Concrete

A second order RSM experiments using three level full factorial CCD was employed to optimize the major parameters influencing the mechanical strength properties of concrete specimen. The parameters, bacterial concentration, curing time and nutritional composition were selected as the numeric factors affecting the mechanical strength properties of the process. 12 runs including 4 centre points were conducted for preparation of specific specimens randomly (Table 3).

### Table 3. Three factor central composite design matrix for Bacterial Concrete

| Run | A     | B     | C     |
|-----|-------|-------|-------|
| 1   | 15    | 28    | 10    |
| 2   | 15    | 28    | 30    |
| 3   | 10    | 15.5  | 20    |
| 4   | 15    | 3     | 30    |
| 5   | 5     | 28    | 30    |
| 6   | 10    | 15.5  | 20    |
| 7   | 15    | 3     | 10    |
| 8   | 5     | 3     | 30    |
| 9   | 10    | 15.5  | 20    |
| 10  | 5     | 28    | 10    |
| 11  | 10    | 15.5  | 20    |
| 12  | 5     | 3     | 10    |

The specimens of bacterial concrete were casted with standard procedures, demoulded after a required curing period and subjected to mechanical strength test. The experimental results were subjected to the second order polynomial model employing multiple regression technique followed by analysis of variance (ANOVA). Coefficient of determination ($R^2$) was assessed to observe the quality of best fit and to explain the variability of the model.

### 3. Results and Discussion

#### Batch Study for the Assessment of Mechanical Properties of Bacterial Concrete

#### 3.1. Effect of Curing Time

The effect of curing time on mechanical properties of bacterial concrete was investigated by varying the curing time as 3, 14, and 28 days at fixed nutritional proportion (10%) and bacterial concentration (5×10^3 cells/mL). Figure 2a represents the effect of curing time on compressive strength of bacterial concrete, which can be compared with conventional concrete. A continuous improvement in the compressive strength was observed with increase in curing time for both the conventional concrete as well as bacterial concrete. However, incorporation of bacteria significantly enhanced the compressive strength of concrete specimen producing a maximum strength of 75.9 MPa. The highest value of compressive strength (69.8 MPa) in case of conventional concrete was achieved is 28 days of curing period, while the similar value of in case of bacterial concrete was observed within 14 days of curing time. From these findings, it is clear that the incorporation of bacteria significantly reduced the curing...
time to produce the similar values of compressive strength. The enhanced strength within the lesser curing period is related to the production of calcite by the utilized bacterial strain leading to the improvement of compressive strength. Similar trends of increase in other strength properties with increase in the curing time were also observed, yielding a maximum flexural strength and split tensile strength of 6 MPa and 5.1 MPa, respectively at 28 days (Figure 2b and Figure 2c). The observed results may be attributed to the fact that introduction of bacteria in the concrete generally improves the strength of concrete specimens by filling the produced calcite into the concrete pores by the process of hydration. However, excess production of calcite may affect the mechanical properties of the concrete [2].

Figure 2. Effect of curing time on; (a) compressive strength, (b) flexural strength, and (c) split tensile strength [10% Nutritional proportion, 5×10³ cells/mL bacterial concentration]

3.2. Effect of bacterial concentration

Mechanical strength properties of different bacterial concrete specimens were measured by varying the bacterial concentration in three steps (5×10³ cells/mL, 10×10³ cells/mL, 15×10³ cells/mL) at constant curing time of 14 days and nutritional proportion of 10%. A continuous increase in the compressive strength, flexural strength and split tensile strength was observed with increase in the bacterial concentration from 5×10³ cells/mL to 15×10³ cells/mL. However, increase in the compressive strength was more prominent as compared to the rest of two properties where only 4-7% of improvement was observed. At extreme bacterial concentration of 15×10³ cells/mL, keeping other two parameters constant, the value of compressive strength, flexural strength and split tensile strength was obtained as 75.8 MPa, 5.7 MPa, and 4.4 MPa respectively (Figure 3). However, the magnitude of these strength properties was way superior to that of conventional concrete at similar conditions of curing period. One likely reason behind this observation is that the initial amount of bacteria incorporated in concrete mixture (5×10³ cells/mL) could also produce the sufficient calcite as higher concentration of bacteria in mixing solution at limited curing period.

Figure 3. Effect of bacterial concentration on mechanical properties of bacterial concrete (14 days Curing time, 10% Nutritional proportion)
3.3. Effect of Nutritional Proportion
The effect of nutritional proportion on the mechanical strength properties of different bacterial concrete specimens were investigated by employing three step variation in the nutritional concentration (10%, 20%, and 30%) keeping rest of the two parameters constant (14 days curing time, 5x10^3 cells/mL bacterial concentration). As can be seen from Figure 4, increase in nutritional proportion did not yield any significant improvement in either of the compressive strength, flexural strength or split tensile strength. At extreme nutritional proportion of 30%, keeping other two parameters constant, the value of compressive strength, flexural strength and split tensile strength was obtained as 71.8 MPa, 5.4 MPa, and 4.2 MPa, respectively (Figure 4). Moreover, the improvement in the mechanical properties with increase in the nutritional proportion was in the range of 2-5%.

Addition of nutritional medium during casting of bacterial concrete specimens ensures the enhancement of calcite formation leading to induce the strength of the specimens. However, in the present experiment, it seems that the initial proportion (10%) itself was sufficient to induce the biocalcification for production of calcite at specified curing period.

![Figure 4](image_url). Effect of nutritional proportion on mechanical properties of bacterial concrete (14 days Curing time, 5x10^3 cells/mL bacterial concentration)

3.4. Statistical model development and effect of parameters
RSM model fitting and statistical analysis was also performed on bacterial concrete with the help of CCD experiments. A total twelve number of experimental runs incorporating various combinations were conducted and corresponding compressive strength values were recorded. The result thus obtained shows a wide range of compressive strength (59.9 MPa to 79.5 MPa), flexural strength (4.3 MPa to 6.22 MPa), and split tensile strength (3.7 MPa to 5.01 MPa), therefore warranted an optimization of the incorporated parameters. The empirical equations for the different mechanical strength properties in terms of coded factors are shown as equation 1, 2 and 3 respectively:

\[
\text{Compressive Strength} = 68.57 + 3.17A + 4.95B + 1.04C + 1.70AB - 0.51AC - 0.052BC + 0.64AB^2 \\
\text{Flexural Strength} = 5.37 + 0.24A + 0.63B + 0.086C - 0.094AB + 0.011AC - 0.026BC - 1.250E - 003AB^2 \\
\text{Split Tensile Strength} = 4.37 + 0.084A + 0.55B + 0.031C - 0.011AB - 6.250E - 003AC + 1.250E - 003BC - 0.011AB^2 \\
\]

Where A, B, and C are the coded terms for the independent variables. The statistical confirmation of the selected model was accomplished employing analysis of variance (ANOVA) and the results are presented in Table 4, 5, 6, respectively for compressive strength, flexural strength, and split tensile strength.
strength. Normally, large F-values, small p-values (<0.05) and R² values approaching towards 1 are considered for the validation of a regression model. As can be seen from the ANOVA Tables, a p-value of <0.0001 (significant) and R² values close to 1 reflect the appropriateness to authenticate the model.

Table 4. ANOVA Results for Compressive Strength

| Source                          | Sum of Squares | DF | Mean Square | F-Value | p-value Prob>F |
|--------------------------------|----------------|----|-------------|---------|----------------|
| Model                          | 313.13         | 7  | 44.73       | 331.44  | 0.0003 Significant |
| A-Bacterial Conc.              | 80.26          | 1  | 80.26       | 594.70  | 0.0002          |
| B-Curing Time                  | 195.82         | 1  | 195.82      | 1450.89 | <0.0001         |
| C-Nutritional Composition AB   | 8.65           | 1  | 8.65        | 64.11   | 0.0041          |
| AB                             | 22.98          | 1  | 22.98       | 170.30  | 0.0010          |
| AC                             | 2.06           | 1  | 2.06        | 15.27   | 0.0298          |
| BC                             | 0.022          | 1  | 0.022       | 0.16    | 0.7132          |
| ABC                            | 3.33           | 1  | 3.33        | 24.66   | 0.0157          |
| Curvature                      | 94.41          | 1  | 94.41       | 699.48  | 0.0001 Significant |
| Pure Error                     | 0.40           | 3  | 0.13        |         |                |
| Cor Total                      | 407.95         | 11 |             |         |                |
| R² = 0.9987                    |                |    |             |         | Adj R² = 0.9957 Adeq Precision = 61.604 |

Table 5. ANOVA Results for Flexural Strength

| Source                          | Sum of Squares | DF | Mean Square | F-Value | p-value Prob>F |
|--------------------------------|----------------|----|-------------|---------|----------------|
| Model                          | 3.80           | 7  | 0.54        | 81.11   | 0.0021 Significant |
| A-Bacterial Conc.              | 0.48           | 1  | 0.48        | 71.03   | 0.0035          |
| B-Curing Time                  | 3.19           | 1  | 3.19        | 476.39  | 0.0002          |
| C-Nutritional Composition AB   | 0.60           | 1  | 0.60        | 8.89    | 0.485           |
| AB                             | 0.070          | 1  | 0.070       | 10.51   | 0.478           |
| AC                             | 1.012E-003     | 1  | 1.012E-003  | 0.15    | 0.7233          |
| BC                             | 5.512E-003     | 1  | 5.512E-003  | 0.82    | 0.4310          |
| ABC                            | 1.250E-005     | 1  | 1.250E-005  | 1.868E-003 | 0.9682          |
| Curvature                      | 0.36           | 1  | 0.36        | 53.46   | 0.0053          |
| Pure Error                     | 0.020          | 3  | 0.0067      |         |                |
| Cor Total                      | 4.18           | 11 |             |         |                |
| R² = 0.9947                    |                |    |             |         | Adj R² = 0.9825 Adeq Precision = 27.102 |
Table 6. ANOVA Results for Split Tensile Strength

| Source                | Sum of Squares | DF | Mean Square | F-Value | p-value | Prob>F |
|-----------------------|----------------|----|-------------|---------|---------|---------|
| Model                 | 2.50           | 7  | 0.36        | 2140.53 | <0.0001 | Significant |
| A-Bacterial Conc.     | 0.056          | 1  | 0.056       | 336.67  | 0.0004  |          |
| B-Curing Time         | 2.43           | 1  | 2.43        | 14586.07| <0.0001 |          |
| C-Nutritional Composition | 7.812E-003  | 1  | 46.87       | 0.0064  |          |          |
| AB                    | 1.012E-003     | 1  | 7.812E-003  | 6.07    | 0.0905  |          |
| AC                    | 3.125E-004     | 1  | 1.012E-003  | 1.87    | 0.2644  |          |
| BC                    | 1.250E-005     | 1  | 5.512E-003  | 0.075   | 0.8020  |          |
| ABC                   | 1.012E-003     | 1  | 1.250E-005  | 6.07    | 0.0905  |          |
| Curvature             | 0.28           | 1  | 0.28        | 1677.02 | <0.0001 | Significant |
| Pure Error            | 5.0E-004       | 3  | 1.667E-004  |         |         |          |
| Cor Total             | 2.78           | 11 |             |         |         |          |
| R² = 0.9998           |                |    |             |         | Adj R² = 0.9993 | Adeq Precision = 117.17 |

The diagnostics of experimental data was employed to strengthen the model validation, which produced the numerous graphical results such as normal plot of residuals, residual vs run number and predicted vs actual plots for compressive strength, flexural strength and split tensile strength (Figure 5).

Figure 5. Model Validation (a. normal plot of residuals, residuals vs run, c. predicted vs actual plots)
All the residuals lie on or close to the normal probability line indicating very small errors in experimentation which affirm the reliability of the model. The plot of residual vs run number indicates that the residual lies well within the acceptable range of ±3, which further strengthen the model validation process. Moreover, the experimental values overlap with the predicted values, indicating the validation of the model validation.

Figure 6. Contour and 3D plots of different combinations for Compressive Strength

3.5 Interactive effect of the factors (variables)

The effects of the selected factors on the mechanical properties of bacterial concrete have been ascertained by the ANOVA. Individual factors A (Bacterial concentration), B (Curing time), and C (nutritional composition) were observed as a significant variable on the basis of p-value (<0.05) for all the studied mechanical properties of bacterial concrete. Moreover, the interactive effect of the model terms AB, AC, and ABC for compressive strength, AB for flexural strength were also appeared to produce the significant effect as compared to other chosen model terms. The interactive effect of the factors was insignificant in case of split tensile strength, since p-value of the two factor model terms as well as three factor model terms are more than 0.05. Therefore, in this case the interactive model terms may be ruled out while deducing the model. The interactive effects of the significant parameters on the mechanical properties was represented by contour and 3D plots (Figure 6), which can be used to recognize the empirical system behaviour in an effective manner. The orange colour region in Figure 6 (on the yellow plane) represents the highest compressive strength. Thus, to achieve it, the range of selected variables should be in this region. The contour and 3D surface plots represent the two factor interaction while the third factor was fixed at centre point. In generally, a high bacterial concentration,
high curing time, and high nutritional composition was appeared to produce high values of mechanical strength properties.

4. Conclusions
The following conclusions are drawn from the comprehensive experimental and statistical investigations on the behavior of bacterial concrete. Mechanical properties of bacterial concrete improved at increasing values of curing time, microbial concentration and nutritional proportional where effect of curing time on improvement of mechanical properties of bacterial concrete appeared as more prominent as compared to the rest parameters. Mechanical properties of concrete have been successfully modelled using empirical equation by optimizing the selected parameters. ANOVA output verified the established model by means of a high correlation coefficient ($R^2 = 0.99$) for all the mechanical properties and non-significant of lack of fit. Further, the interactive effect of selected parameters was also explored and presented with the help of 3D surface as well as contour plots. The interactive effect of the model terms AB, AC, and ABC for compressive strength, AB for flexural strength were appeared to produce the significant effect as compared to other chosen model terms. Overall, the application of culture broth of *Bacillus subtilis* directly into fresh concrete at different nutritional proportion improved the strength of concrete considerably.

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