International Journal of Engineering Applied Sciences and Technology, 2019
Vol. 4, Issue 7, ISSN No. 2455-2143, Pages 245-251
Published Online November 2019 in IJEAST (http://www.ijeast.com)

GROWTH MEDIUM OPTIMIZATION FOR CALCITE PRECIPITATION USING RESPONSE SURFACE METHODOLOGY

J. Neena Priya, V. Nithyakalyani
Department of Microbiology,
Dr. MGR Janaki College of Arts & Science for Women,
Chennai, India

Abstract - The nutritional medium that stimulated the production of carbonate using nitrogen cycle metabolic pathways was explored. Different media used for bacterially induced precipitation of calcium carbonate were brought out for the optimization of a nutrient medium and limit the frequency of feeding to meet industrial economic constraints. The ability of cultures to grow and produce without expensive growth factors also should be assessed. The medium optimization uses nutrient consumption as a foundation for medium formulations stoichiometrically. Statistical experimental designs can aid in interpretation of results and help economize the number of experiments required. There are only very few studies related to optimization of the medium for carbonate precipitation. In this study, the maximum calcite producing bacterial strains *Bacillus sp* and *Pseudomonas sp* were selected from previous screening study (Priya, J. N *et al*., 2017) and compared the results obtained by laboratory experiments with experimental design CCD of Response Surface Methodology.

Keywords - RSM, Optimization, calcite precipitation, nutrient medium

I. INTRODUCTION

There are many studies in biomineralization of prokaryotes (Rivadeneyra, M. A *et al*., 2010; Konhauser, K., & Riding, R, 2012; Anbu, P *et al*., 2016; Bindschedler, S *et al*., 2016) but less data is available for the medium employed for the calcite precipitation. Only few very studies have been noted in the optimization of the medium (Duan, T., & Zhu, W. K., 2012). The main goal of this study is to optimize the medium for the calcite precipitation. A useful start to medium optimization is to focus on an initial basal medium contains carbohydrates, amino acids, vitamins, minerals, lipids and growth factors that can be evaluated one at a time or in concert. Screening experimental designs are useful for determining which components have significant effects on cell growth, viability, and productivity with these components at low and high concentration ranges (Jerums, M., & Yang, X., 2005).

Response surface methodology (RSM) proposed an effective statistical method to be applied for defining the individual character of each factor, its antagonistic and opposed effects (Steinberg, D. M., & Bursztyn, D *et al*., 2010). It is widely used to optimize bacterial growth, enzymatic and chemical reactions. The specific consumption rates can be calculated and the concentrations of each component of the medium adjusted in the formulation to reflected their actual use. The best calcite producing strains such as B7 strain of *Bacillus sp* and P9 of *Pseudomonas sp* for its maximum EPS content and highest enzyme activity of urease and carbonic anhydrase analysed in the previous study (Priya, J. N *et al*., 2017) were selected to perform optimization study (Gorlach-Lira, K *et al*., 2010). However, this study describes the central composite design (CCD) of the RSM component used to derive at the achieve maximum concentration of calcite under different parameters of growth such as carbon source glucose, nitrogen source beef extract, calcium acetate, temperature and pH.

II. MATERIALS & METHODS

A. Laboratory method

Calcite precipitating B4 media constitutes of glucose, beef extract, calcium acetate and agar was used for calcite precipitation by the bacterial strains. The selected strains B7 of *Bacillus sp* and P9 of *Pseudomonas sp* were further optimized to achieve maximum concentration of calcite. The five factors influenced in the calcite precipitation that includes carbon source glucose, nitrogen source beef extract,

International Journal of Engineering Applied Sciences and Technology, 2019
Vol. 4, Issue 7, ISSN No. 2455-2143, Pages 245-251
Published Online November 2019 in IJEAST (http://www.ijeast.com)

calcium acetate, temperature and pH were used in different concentrations to determine maximum calcite production. The carbon source glucose concentration ranged from 0.3 to 0.7g, nitrogen source beef extract from 0.2 to 0.6 g, calcium acetate from 1 to 1.9 g, temperature from 30ºC to 37ºC and pH from 7 to 10 was used to stimulate the bacterial growth to produce calcite and quantified by EDTA titration method in the laboratory experiment (Achal, V *et al*., 2009; Park, S. J *et al*., 2010 Gavimath, C. C *et al*., 2012).

B. Experimental design

The optimization of the interaction of above mentioned five variables was performed by using RSM with the experimental design (Raharjo, S *et al*., 2016) as mentioned in the Table:1 using Statease software Design – Expert (version. 11).

Table:1 Factors and its level ranges in the experimental design

Factors	Low level range	High level range
Glucose	0.3	0.7
Beef extract	0.2	0.6
Calcium acetate	1	1.9
Temperature	30	37
pH	7	10

Central Composite designs (CCD) used in this experimental study are based on two-level factorial designs, augmented with center and axial points to fit quadratic models. The number of runs required for the numeric factors will be multiplied by the number of categorical combinations about 53 runs for *Bacillus* (B7) strain and *Pseudomonas* (P9) strain respectively.

III. RESULTS

The result of the Response surface methodology by the design type of central composite with 53 runs or observations was tabulated and highlighted with actual and predicted values of the experiment as in Table:2 and Table:3 for *Bacillus* (B7) and *Pseudomonas* (P9) strain respectively.

Table: 2 RSM report of *Bacillus* (B7) strain

Run Order	Actual Value	Predicted Value[1]	Residual
1	10.00	9.97	0.0279
2	17.00	17.27	-0.2725
3	21.00	19.51	1.49
4	19.00	18.26	0.7433
5	15.00	17.27	-2.27
6	10.00	11.23	-1.23
7	18.00	19.37	-1.37
8	20.00	18.87	1.13
9	15.00	14.23	0.7680
10	20.00	18.87	1.13
11	10.00	11.23	-1.23
12	22.00	21.77	0.2333
13	10.00	9.97	0.0279
14	10.00	11.23	-1.23
15	10.00	9.97	0.0279
16	17.00	17.27	-0.2725
17	17.00	17.27	-0.2725
18	17.00	17.27	-0.2725
19	19.00	19.37	-0.3708
20	19.00	18.26	0.7433
21	13.00	14.23	-1.23
22	18.00	19.37	-1.37
23	14.00	14.23	-0.2320
24	17.00	17.27	-0.2725
25	22.00	21.77	0.2333
26	20.00	18.87	1.13
27	10.00	11.23	-1.23
28	21.00	19.51	1.49
29	22.00	21.77	0.2333
30	13.00	14.23	-1.23
31	20.00	18.87	1.13
32	17.00	17.27	-0.2725
33	19.00	18.26	0.7433
34	19.00	18.26	0.7433
35	19.00	19.37	-0.3708
36	21.00	19.51	1.49
37	10.00	9.97	0.0279
38	22.00	21.77	0.2333
39	17.00	17.27	-0.2725
40	21.00	19.51	1.49
41	18.00	18.48	-0.4814
42	18.00	20.18	-2.18
43	17.00	16.39	0.6126
44	15.00	16.39	-1.39
45	17.00	16.39	0.6126
46	15.00	14.29	0.7067
47	15.00	16.39	-1.39
48	18.00	21.01	-3.01
49	17.00	16.39	0.6126
50	7.00	5.56	1.44
51	15.00	11.77	3.23
52	17.00	16.39	0.6126
53	17.00	16.39	0.6126

[1] Predicted values include block corrections.

International Journal of Engineering Applied Sciences and Technology, 2019
Vol. 4, Issue 7, ISSN No. 2455-2143, Pages 245-251
Published Online November 2019 in IJEAST (http://www.ijeast.com)

Table: 3 RSM report of *Pseudomonas* (P9) strain

Run Order	Actual Value	Predicted Value[1]	Residual
1	9.00	10.33	-1.33
2	15.00	14.05	0.9500
3	12.00	10.33	1.67
4	12.00	14.05	-2.05
5	19.00	17.91	1.09
6	13.00	12.87	0.1259
7	19.00	17.91	1.09
8	17.00	15.09	1.91
9	19.00	17.91	1.09
10	12.00	14.05	-2.05
11	19.00	17.91	1.09
12	10.00	10.33	-0.3251
13	10.00	12.87	-2.87
14	17.00	15.09	1.91
15	12.00	14.05	-2.05
16	13.00	12.87	0.1259
17	13.00	12.87	0.1259
18	12.00	14.05	-2.05
19	10.00	10.33	-0.3251
20	14.00	15.09	-1.09
21	10.00	10.33	-0.3251
22	17.00	15.09	1.91
23	9.00	10.33	-1.33
24	19.00	17.91	1.09
25	19.00	17.91	1.09
26	19.00	17.91	1.09
27	12.00	14.05	-2.05
28	12.00	14.05	-2.05
29	17.00	15.09	1.91
30	13.00	12.87	0.1259
31	12.00	10.33	1.67
32	19.00	17.91	1.09
33	13.00	12.87	0.1259
34	14.00	15.09	-1.09
35	17.00	15.09	1.91
36	13.00	12.87	0.1259
37	16.00	15.09	0.9126
38	9.00	10.33	-1.33
39	13.00	12.87	0.1259
40	12.00	14.05	-2.05
41	11.00	12.78	-1.78
42	14.00	18.44	-4.44
43	14.00	12.62	1.38
44	14.00	12.62	1.38
45	14.00	12.62	1.38
46	11.00	6.79	4.21
47	14.00	12.62	1.38
48	13.00	12.62	0.3846
49	13.00	12.62	0.3846
50	13.00	12.62	0.3846
51	11.00	12.45	-1.45
52	9.00	12.62	-3.62
53	13.00	12.62	0.3846

[1] Predicted values include block corrections.

The highlighted report of *Bacillus* (B7) strain and *Pseudomonas* (P9) strain showed much similar identity in the actual and predicted values in the calcite precipitation. In the Table:2 run order 12 and 38 showed 22% in the actual value and 21.77% in the predicted value with a leverage of 0.189 for *Bacillus* (B7) strain. Both actual and predicted values were much closer in the laboratory and experimental design. In the Table:3 run order 24, 25 and 26 showed 19% in the actual value and 17.91% in the predicted value with a leverage of 0.102 for the *Pseudomonas* (P9) strain. Both the values were not that closer but comparatively had much less difference between them than other run order combinations. Analysis of variance (ANOVA) for the calcite produced was presented in Table: 4. The significant effect of F and p-value. Here p-value is the probability of rejecting for the null hypothesis of the study.

Table: 4 ANOVA for calcite forming strains

Source	***Bacillus* (B7) strain**	***Pseudomonas* (P9) strain**
Sum of Squares	697.91	318.06
Degree of freedom	7	3
Mean Square	99.70	106.02
F value	63.80	35.12
P value	< 0.0001	< 0.0001
Actual R^2	0.9103	0.6870
Predicted R^2	0.8276	0.5946

Figure:1, 2 and 3 showed the curvature of *Bacillus* (B7) strain in a 3D Surface Plot depicting the relationship between the model equations with the response of calcite production. The increase in (X1) calcium acetate and temperature (X2) resulted in the increased calcite production. The increase in factors such as temperature (X1) and pH (X2) had lesser effect in the calcite production while the other two factors such as glucose and beef extract did not contribute much on the calcite production.

Figure: 3, 4 and 5 showed the curvature of *Pseudomonas* (P9) strain in a 3D Surface Plot depicting the relationship between the model

International Journal of Engineering Applied Sciences and Technology, 2019
Vol. 4, Issue 7, ISSN No. 2455-2143, Pages 245-251
Published Online November 2019 in IJEAST (http://www.ijeast.com)

equations with the response of calcite production. The increase in (X1) calcium acetate and temperature (X2) resulted in the increased calcite production. However, factors temperature (X1) and pH (X2) showed less effect in the calcite production whereas other factors glucose and beef extract did not show much effect on the calcite production. As per the result of RSM obtained for optimization analysis of calcite production it was found to be favourable with the laboratory results. Among the factors used in the study, carbon and nitrogen sources used in the formulation of the medium differed in studies reported.

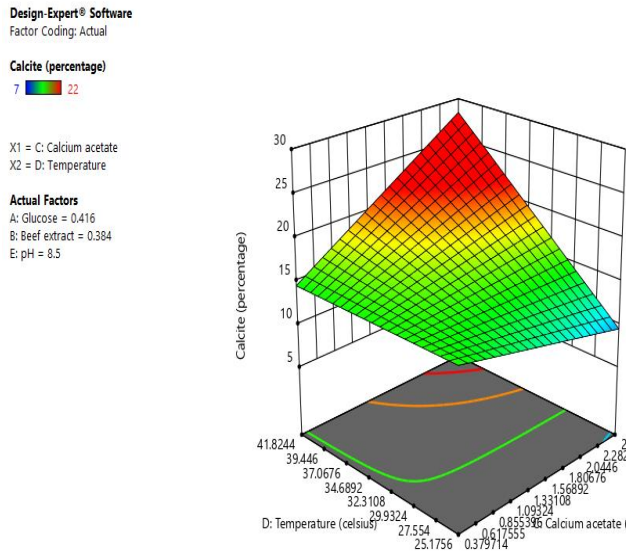

Figure: 1 3D Surface plot for calcium acetate and temperature of *Bacillus* (B7) strain

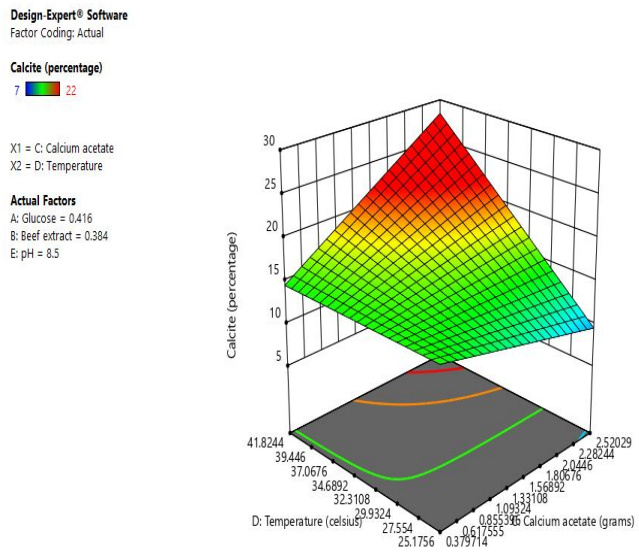

Figure: 2 3D Surface plot for calcium acetate and pH of *Bacillus* (B7) strain

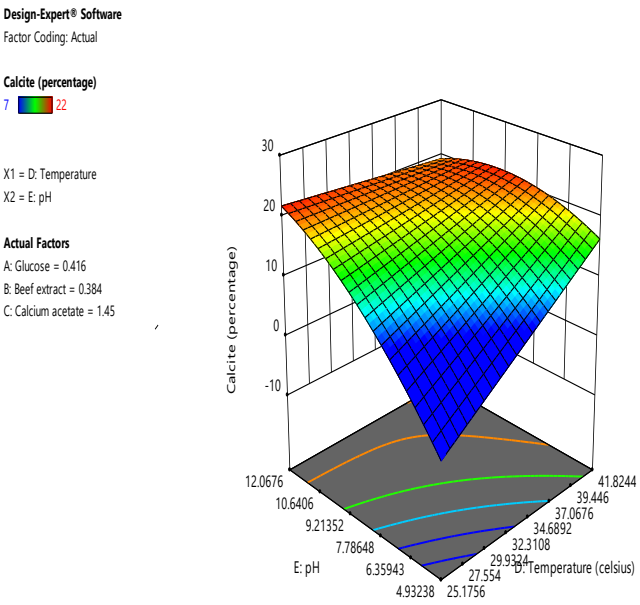

Figure: 3 3D Surface plot for temperature and pH of *Bacillus* (B7) strain

International Journal of Engineering Applied Sciences and Technology, 2019
Vol. 4, Issue 7, ISSN No. 2455-2143, Pages 245-251
Published Online November 2019 in IJEAST (http://www.ijeast.com)

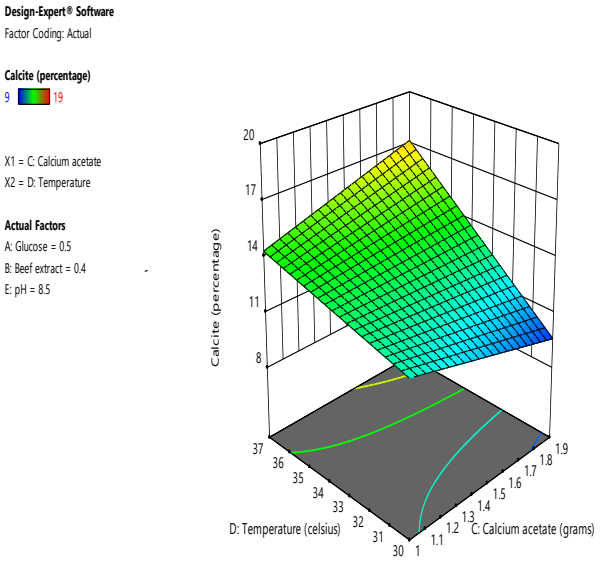

Figure: 4 3D Surface plot for calcium acetate and temperature of *Pseudomonas* (P9) strain

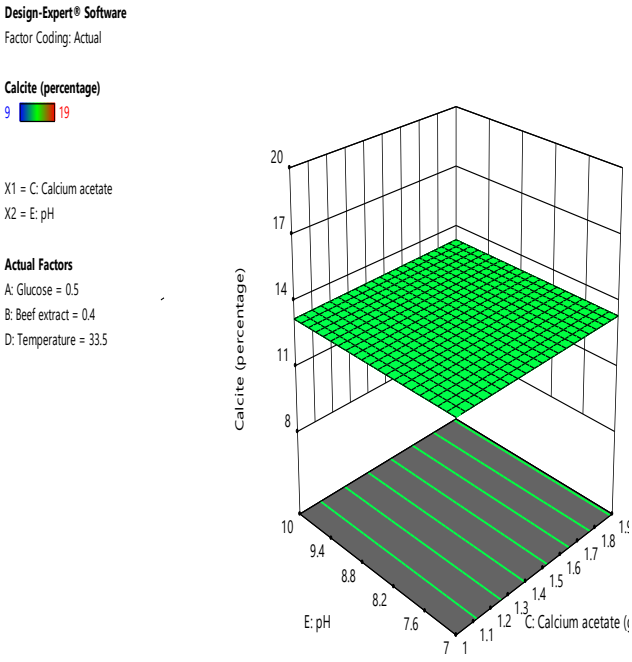

Figure: 5 3D Surface plot for Calcium acetate and pH of *Pseudomonas* (P9) strain

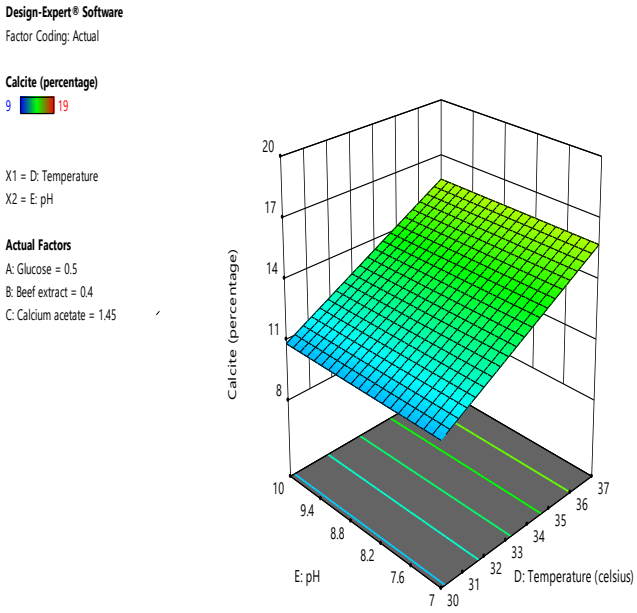

Figure: 6 3D Surface plot for Temperature and pH of *Pseudomonas* (P9) strain

IV. DISCUSSION

Calcite precipitation by bacterial isolates *Bacillus thuringiensis* and *B. pumilis* were cultured on B-4 agar medium mediated the calcite precipitation (Baskar, S *et al*., 2006). In this study, B-4 medium was used without any modification in the experiment. The values for each composition ranged from minimum to maximum values contributing to calcite production (Achal, V *et al*., 2009; Park, S. J *et al*., 2010 Gavimath, C. C *et al*., 2012).

There are few studies which concentrated on calcium source as an important factor used in the B4 medium in calcite precipitation. In a study of microbiologically induced $CaCO_3$ precipitation by *Sporosarcina pasteurii* ATCC 11859, effect of two different types of calcium sources such as calcium nitrate as a representative of inorganic calcium source and calcium lactate as an organic calcium source was investigated. It was substituted instead of calcium chloride since chloride ions might be detrimental for the concrete reinforcement (Xu, J *et al*., 2015). Hence in the present study the factor calcium acetate used in the B4 medium remain unchanged as it played a major role in the calcite production.

The bacterial strains after a complete analysis of enzyme and EPS profile, growth medium for calcite precipitation suggested a need for optimization studies (Jerums, M., & Yang, X., 2005; Lv, G. Y *et al*., 2008; Duan, T., & Zhu, W. K., 2012; Raharjo, S *et al*., 2016). The statistically RSM have implied the correlation between five variables carbon source glucose, nitrogen

source beef extract, calcium acetate, temperature and pH (Xu, J *et al*., 2015). Among the variables after analysis it showed a significant increase in calcite production with strong influence of temperature and calcium acetate, but not much with the other three variables, highlighted the values of actual and predicted values for both the bacterial strains with respect to the calcite production and these findings add to the strong significance of calcite precipitation and growth medium statistically as well.

Response surface methodology was the approach used to optimize medium constituents for enzymatic production by *Pseudomonas sp* Zjwp-14 strain. It allowed rapid screening of the important factors that are influential and developed a polynomial model. Plackett-Burman design experiment was used to investigate quantitative effects for four variables on enzyme activity (Lv, G. Y *et al*., 2008). Central Composite designs (CCD) was applied in this study to optimize the media constituents for both *Bacillus* (B7) and *Pseudomonas* (P9) strain in the calcite precipitation.

An easy High throughput screening (HTS) strategy was developed for the screening of $CaCO_3$ producing microorganisms after UV treatment. About 3000 isolates were tested for its mineralizing capacity and *Bacillus pseudofirmus* DSM8715 strain was found to exhibit 92.67% of calcium mineralizing activity by HTS strategy in a much less time of three weeks. It employed O-Cresolphthalein Complexone (OCPC) method for HTS strategy (Zhang, J. L *et al*., 2013). However, the present study unable to use HTS strategy as the compositions were expensive and the primary focus of the study is to use cost effective methods. The calcite forming bacteria were screened by the percentage of calcite produced by EDTA titration method (Achal, V *et al*., 2009).

Duan, T., & Zhu, W. K. (2012) studied the effect of temperature, pH, precipitation time, concentration of the reactants, crystal formation additive on the yield of calcium carbonate precipitation by purchased ATCC strain *Bacillus pasteurii* through orthogonal test. The parameters included were pH from 7 to 10, temperature ranging from 35ºC to 50ºC, for a time period of four days, different concentrations of Mg^{2+} from 0.04 to 0.07mol/L and the concentration of Ca^{2+} from the range 1 to 2.5 mol/L. He reported that temperature had greater impact than concentration of Mg^{2+} than pH than precipitation time than concentration of Ca^{2+}. The optimal combination for the largest yield of calcium carbonate precipitation was pH of 8 with 0.05 mol/L Mg^{2+} concentration, 1.5mol/L Ca^{2+} concentration, 40ºC temperature and 3days of precipitation time. Based on the above mentioned study, the present work formulated the five variables with the minimum and maximum ranges. The temperature ranges between 30ºC to 37ºC, pH between 7 and 10, calcium source of calcium acetate was used instead of calcium chloride, carbon sources glucose same as the study but protein source beef extract was used instead of peptone in the experimental design thereby contributing in calcite precipitation.

As B4 medium was not only used in the microbiologically induced calcite precipitation but could be extended to other strains isolated the growth of isolates from natural environments which was investigated in two domesticated laboratory strains. The buffering capacity of the medium was compromised to investigate the calcite precipitation and it did play a major role in the process of precipitation. Both the pH and buffering capacity contributed in the mineral precipitation (Marvasi, M *et al*., 2012). Hence the current study had an effective pH range for calcite production between 7 and 10 by not much modifying the buffering capacity and the pH variable not less than 7 and not more than 10 was maintained thus proved the effectiveness of pH in the mineralization experiment.

V. CONCLUSION

Thus selected bacterial strain of genus *Bacillus* (B7) and *Pseudomonas* (P9) in the previous study (Priya JN et al., 2017) were subjected to optimization of five factors influencing *in vitro* calcite precipitation. The highest calcite produced by *Bacillus* (B7) strain was 22% as the actual value and the predicted value was 21.77% and lesser residual value of 0.23 with much lesser difference. However, the difference in the glucose did not affect the maximum calcite production. The highest calcite produced by *Pseudomonas* (P9) strain was 19% as the actual value and with slightly increased difference in the predicted value of 17.91% with much higher residual value of 1.09. It might be the pigment producing ability of *Pseudomonas sp* that interferes with the calcite production with much higher residual value. Considering the effect of these parameters on the basis of data obtained, it was thus proved both in laboratory and experimental designs, B4 medium as an efficient medium to sustain calcite forming bacteria in a natural environmental conditions.

VI. REFERENCES

[1] Achal, V., Mukherjee, A., Basu, P. C., & Reddy, M. S. (2009). Lactose mother liquor as an alternative nutrient source for microbial concrete production by

Sporosarcina pasteurii. *Journal of industrial microbiology & biotechnology*, *36* (3), 433-438.
[2] Achal, V., Mukherjee, A., Basu, P. C., & Reddy, M. S. (2009). Strain improvement of *Sporosarcina pasteurii* for enhanced urease and calcite production. *Journal of industrial microbiology & biotechnology*, *36* (7), 981-988.
[3] Anbu, P., Kang, C. H., Shin, Y. J., & So, J. S. (2016). Formations of calcium carbonate minerals by bacteria and its multiple applications. *Springerplus*, *5*(1), 250.
[4] Baskar, S., Baskar, R., Mauclaire, L., & McKenzie, J. A. (2006). Microbially induced calcite precipitation in culture experiments: possible origin for stalactites in Sahastradhara caves, Dehradun, India. *Current science*, 58-64.
[5] Bindschedler, S., Cailleau, G., & Verrecchia, E. (2016). Role of fungi in the biomineralization of calcite. *Minerals*, *6* (2), 41.
[6] Duan, T., & Zhu, W. K. (2012). Optimization of Calcium Carbonate Precipitation for *Bacillus pasteurii*. In *Applied Mechanics and Materials*, *178*, 676-679. Trans Tech Publications.
[7] Gavimath, C. C., Mali, B. M., Hooli, V. R., Mallpur, J. D., Patil, A. B., Gaddi, D & Ravishankera, B. E. (2012). Potential application of bacteria to improve the strength of cement concrete. *Int. J. Adv. Biotechnol. Res*, *3* (1), 541-544.
[8] Gorlach-Lira, K., Pedroza, M. L. V., Burdziej-Pokojska, A., Rozycki, H., & Dahm, H. (2010). Response surface analysis on the effect of temperature and pH on Growth and proteolytic activity of thermophilic *Bacillus sp*. *Brazilian Archives of Biology and Technology*, *53* (5), 1067-1072.
[9] Marvasi, M., Visscher, P. T., Perito, B., Mastromei, G., & Casillas-Martínez, L. (2010). Physiological requirements for carbonate precipitation during biofilm development of *Bacillus subtilis* etfA mutant. *FEMS microbiology ecology*, 71 (3), 341-350.
[10] Jerums, M., & Yang, X. (2005). Optimization of cell culture media. *BioProcess Int*, *3* (6), 38-44.
[11] Konhauser, K., & Riding, R. (2012). Bacterial biomineralization. *Fundamentals of Geobiology*, 105-130.
[12] Lv, G. Y., Wang, P., He, J. Y., & Li, X. N. (2008). Medium optimization for enzymatic production of L-cysteine by *Pseudomonas sp*. Zjwp-14 using response surface methodology. *Food Technology and Biotechnology*, *46* (4), 395-401.
[13] Park, S. J., Park, Y. M., Chun, W. Y., Kim, W. J., & Ghim, S. Y. (2010). Calcite-forming bacteria for compressive strength improvement in mortar. *Journal of microbiology and biotechnology*, *20* (4), 782-788.
[14] Priya, J. N., & Kannan, M. (2017). Effect of Carbonic Anhydrase and Urease on Bacterial Calcium Carbonate Precipitation. *International Journal of Pharma and Bio Sciences*, *8*(3), 609-614.
[15] Raharjo, S., Muryanto, S., Jamari, J., & Bayuseno, A. P. (2016). Modeling and optimization of $CaCO_3$ precipitated from laminar-flow water in the presence of citric acid at an elevated temperature. *International Journal of Applied Engineering Research*, 11 (15), 8533-8539.
[16] Rivadeneyra, M. A., Martín-Algarra, A., Sánchez-Román, M., Sánchez-Navas, A., & Martín-Ramos, J. D. (2010). Amorphous Ca-phosphate precursors for Ca-carbonate biominerals mediated by *Chromohalobacter marismortui*. *The ISME journal*, *4* (7), 922-932.
[17] Steinberg, D. M., & Bursztyn, D. (2010). Response Surface Methodology in biotechnology. *Quality Engineering*, *22* (2), 78-87.
[18] Xu, J., Du, Y., Jiang, Z., & She, A. (2015). Effects of Calcium Source on Biochemical Properties of Microbial $CaCO_3$ Precipitation. *Frontiers in microbiology*, *6*.
[19] Zhang, J. L., Deng, X., Feng, X., Han, N. X., & Jonkers, H. M. (2013). Improvement on bacteria-induced calcium mineralization ability of *Bacillus pseudofirmus* by an integrated high-throughput screening strategy. In *ICSHM 2013: Proceedings of the 4th International Conference on Self-Healing Materials, Ghent, Belgium*, Magnel Laboratory for Concrete Research.