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Enhancing urease enzyme production of *Bacillus subtilis* OQ119616 by different calcium salts using Box–Behnken statistical experimental design

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ABSTRACT

Urease production by bacteria represents the key elements of several environmental processes including wastewater treatment, fertilizers management, and bio-cement processes. Maximizing urease production from the microbial strains is considered the first step for efficient utilization of the product. *Bacillus subtilis* OQ119616 was recovered from Sahel Salim soil sample and identified phenotypic and genotypic using 16SrRNA gene sequence. Box–Behnken statistical experimental design with 13- runs were utilized to optimize and evaluate the interaction effects, main effects, and the quadratic effects of four different Ca-salts on the OD (600 nm) and urease specific activity by *B. subtilis* in liquid fermentation. The maximum specific activity of urease enzyme for calcium chloride was 0.540 (predicted value 0.558) obtained in run (9) using urea 20 g/l (A), calcium chloride 20 g/l (B), and pH 6 (C), while calcium nitrate was 0.441 (predicted value 0.418) obtained in run (4) using urea 30 g/l (A), calcium nitrate 10 g/l (B), and pH 6 (C). For calcium acetate was 0.435 (predicted 0.430) obtained in run number (12) using urea 10 g/l (A), calcium acetate 1.5 g/l (B), and pH 6 (C), while calcium citrate was 0.653 (predicted value 0.666) obtained in run number (10) using urea 30 g/l (A), calcium citrate 100 M (B), and pH 8 (C). R^2 values of urease specific activities were 0.979 (calcium chloride), 0.981 (calcium nitrate), 0.973 (calcium acetate), and 0.981 (calcium citrate) which indicated that the whole variations were explained highly and accurately by the statistical model. Optimizing urease production using statistical designs represents a rapid, efficient, and cost-effective technique.

INTRODUCTION

Through mechanisms including enzyme-induced carbonate precipitation and microbially induced calcium carbonate precipitation, ureases—a common metalloenzyme (EC 3.5.1.5) play a critical role in the creation of bio-cement [1]. Using urease, these techniques hydrolyze urea to produce ammonia and carbonate ions, which then react with calcium ions to generate precipitates of calcium carbonate. This method improves the mechanical qualities of soil or sand particles and offers a sustainable substitute for conventional cement [2]. In the last several years, the use of biological technology in geotechnical engineering has gained more attention [3]. One method that has shown some promise is the use of bioactivity in sand cementation via calcium carbonate precipitation, or more precisely, microbially induced calcite precipitation (MICP). The MICP type that is most frequently utilized is passive precipitation. In this process, the system's pH is altered by bacterial activity, typically urea hydrolysis, which results in the production of carbonate ions. Then, inside the soil pore space, CaCO3 chemical precipitation takes place when calcium ions are present. The urease enzyme, which is produced by certain bacteria, raises the pH of the system and produces carbonate ions. The process is started by the hydrolysis of urea:

$$CO(NH_2)^2+3H_2O\rightarrow 2NH_4^+$$

Calcium carbonate precipitates at an increasing pace until pH 9 [4,5] when pH rises over 8.3, at which point the precipitation tends to return pH to neutral. However, the actual final pH of the solution is determined by substrate concentrations and reaction rates [6].

$$HCO_3^- \rightarrow CO_3^{-2}+H^+$$

$$Ca^{+2}+CO_3^{-2}\rightarrow CaCO_3\downarrow$$

When the bacteria have hydrolyzed all of the available urea, created carbonate, and present calcium precipitate as calcium carbonate at the desired volume, the MICP process is said to be 100% efficient. On the other hand, calcium and urea, the two main chemicals introduced, precipitate as calcium carbonate, which is the definition of chemical efficiency. Thus, unless precipitation occurs in the intended site, a 100% chemical efficiency does not guarantee a 100% overall process efficiency.

MATERIALS AND METHODS

Sample Collection and Isolation of MICB Bacteria:

In order to isolate microorganisms, soil samples were gathered from Sahel Salim in the Assiut Governorate of Egypt. The isolation of microorganisms was evaluated using serial dilution. The spread plate method was used to plate the samples onto nutrient agar plates. Agar (1.5%), sodium chloride (0.8%), peptone (0.5%), and beef extract (0.3%) make up nutrition agar plates. For a whole day, the plates were incubated at 35°C. Stored at 4°C, the isolated strains were kept on nutrient agar slants. To produce single colonies from each sample, the isolates were purified by streaking them onto solid media many times. Subculturing onto these media further purified a single colony. For additional research, the developing bacteria will be maintained in slants.

Bacterial identification

Classification techniques described by Harrigan [7] were applied to the isolated bacteria. Based on the chemical and physical characteristics of their cell walls, bacterial species can be easily divided into two major groups using the Gram stain: Gram-positive and Gram-negative.

Biochemical reaction tests for bacteria

The UK Standards for Microbiology Investigation and Bergey's Manual keys were used to characterize and identify the acquired isolate. The catalase enzyme was used to assess our isolate's identity. One of the oxidative byproducts of aerobic carbohydrate metabolism is hydrogen peroxide. The citrate test makes use of starch hydrolysis and Simmon's citrate test. Hugh-Leifson semi-solid medium was utilized to distinguish between the fermentation and oxidation of glucose [8]. Frazier gelatin agar was utilized for gelatin hydrolysis [9]. reduction of Nitrate [10]. production of acid from carbohydrates; lactose, glucose, sucrose, and other carbohydrates were added to the basal medium at a rate of 0.5% w/v. Utilization of various carbon sources: 0.5% (w/v) of various carbon sources were added to the basal medium [11].

Experimental Set up

Statistical optimization of urease enzyme production by *B. subtilis* using four calcium salts

To optimize and assess the main effects, interaction effects, and quadratic effects of the tested variables on the urease activity and optical density (OD) (600 nm) of *B. subtilis* in liquid fermentation employing the four distinct Ca-salts, a Box–Behnken statistical experimental design

was employed. For 72 hours, *B. subtilis* was cultivated in 100 milliliters of minimum medium (5 grams of peptone and 5 grams of beef extract per liter) in a rotatory shaker incubator until it reached 10^9 cells ml^{-1}. The quadratic answers and the second order polynomial models were investigated using a 13-run, three-parameter, three-level statistical design. Tables 2 and 3 were used to clear the design's independent and dependent variables. Three levels (-1, 0, +1) were used to test the following three parameters: pH (6, 7, 8) (C), calcium salts (calcium citrate (50, 100, 150 M), calcium nitrate (5, 10, 15 g/l) (B), calcium acetate (1, 1.5, 2 g/l) (B), calcium chloride (10, 15, 20 g/l) (B)), and urea (10, 20, 30 g/l) (A). The quadratic equation (1) produced the non-linear quadratic statistical model:

$$Y= \beta_0+ \sum\beta_i x_i +\sum\beta_{ii} x_i^{\,2}+ \sum\beta_{ij}x_{ij} \qquad \textbf{(1)}$$

The intercept is denoted by β0, the linear impact by βi, the squared impact by βii, the interaction impact by βij, and the independent levels of variables by xij. Y represents the expected values of OD or urease enzyme of the studied bacteria. Response surface (3D) plots, comparison curves between actual and expected values, and an analysis of the design's and the data's statistical efficiency were all used to show the correlations between the variables.

Analysis

In a rotatory shaker incubator, we created 13 conical flasks with 100 ml of minimum medium (three distinct urea, calcium salt, and pH concentrations) and 1 ml of *Bacillus subtilis* inoculum for a duration of 72 hours. Every day, we tested the O.D. at 660 nm using a spectrophotometer after taking 2 ml. The urease enzyme was measured using a spectrophotometer set at 450mm and 1 ml of minimum medium and 1 ml of Stuart's Urea Broth (yeast extract 0.1 g potassium phosphate monobasic 9.1 g potassium phosphate, dibasic 9.5 g, phenol red 0.01 g) in 1L [12].

RESULTS

Bacterial identification

According to the findings, our isolate may be classified as a *Bacillus* sp. According to Barrow and Feltham's instructions, morphological and biochemical tests revealed that our isolate tested positive for urease synthesis, catalase, oxidase test, nitrate reduction, fermentation of glucose, mannitol, and sucrose, as well as urease activity synthesis and Gram stain. According to

molecular identification, the studied strain was identified as *Bacillus subtilis* OQ119616 (data not published).

Statistical optimization of urease enzyme production by *Bacillus subtilis* using four calcium salts

Box–Behnken statistical design was stated to optimize and evaluate the main effects, interaction, and the quadratic effects of the tested variables on the OD (600 nm) and urease production by *Bacillus subtilis* in liquid fermentation using expired four different Ca-salts. Three-parameter (Urea (10, 20, 30 g/l) (A), Calcium chloride (10, 15, 20 g/l) (B), Calcium nitrate (5, 10, 15 g/l) (B), Calcium acetate (1, 1.5, 2 g/l) (B), Calcium citrate (50, 100, 150 M), and pH (6, 7, 8) (C)), three-level (-1, 0, +1) statistical design with 13-runs was sets **as cleared in tables (1&2)**. Bacterial growth was expressed as OD at 600 nm, and urease activity was calculated as specific activity. The predicted values of the tested parameters during the 13- runs were calculated through the design second-order polynomial **equation (1);** Bacterial OD (600nm), and Urease specific activity predicted values were calculated by the **equation 2&3** (calcium chloride), **4&5 (**calcium nitrate), **6&7** (calcium acetate), and **8&9** (calcium citrate) as following:

Bacterial OD (600nm) = 1.12 + (-0.0069) A + (-0.0096) B + (-0.1468) C + ((-0.028) A * B) + ((-0.0447) A * C) + ((-0.0922) B * C) + (-0.0595) A^2 + (0.0265) B^2 + (-0.0865) C^2 **(2)**

Urease specific activity = 0.5314 + (0.0023) A + (-0.0295) B + (-0.0522) C + ((-0.0704) A * B) + ((0.0294) A * C) + ((-0.1054) B * C) + (-0.1209) A^2 + (-0.0183) B^2 + (-0.0825) C^2 **(3)**

Bacterial OD (600nm) = 0.9665 + (-0.0151) A + (-0.0487) B + (-0.2176) C + ((-0.0275) A * B) + ((0.0006) A * C) + ((-0.0251) B * C) + (-0.0062) A^2 + (0.0255) B^2 + (-0.2054) C^2 **(4)**

Urease specific activity = 0.4119 + (-0.0004) A + (-0.0403) B + (-0.0985) C + ((-0.0043) A * B) + ((-0.0112) A * C) + ((0.0113) B * C) + (0.0005) A^2 + (-0.0295) B^2 + (-0.1032) C^2 **(5)**

Bacterial OD (600nm) = 0.932 + (-0.081) A + (-0.0289) B + (-0.0818) C + ((0.0439) A * B) + ((0.0267) A * C) + ((-0.0789) B * C) + (0.0725) A^2 + (0.0347) B^2 + (-0.0028) C^2 **(6)**

Urease specific activity = 0.3227 + (-0.0185) A + (-0.0042) B + (-0.0608) C + ((-0.0238) A * B) + ((0.0697) A * C) + ((-0.0234) B * C) + (-0.0616) A^2 + (-0.0215) B^2 + (0.02) C^2 **(7)**

Bacterial OD (600nm) = 0.778 + (-0.0246) A + (-0.0426) B + (-0.1227) C + ((-0.0819) A * B) + ((-0.077) A * C) + ((-0.0194) B * C) + (0.0141) A^2 + (-0.0627) B^2 + (-0.0579) C^2 **(8)**

Urease specific activity = 0.4418 + (0.0804) A + (0.0336) B + (0.0009) C + ((-0.0174) A * B) + ((0.0593) A * C) + ((-0.1046) B * C) + (0.0237) A^2 + (-0.0288) B^2 + (-0.0608) C^2 **(9)**

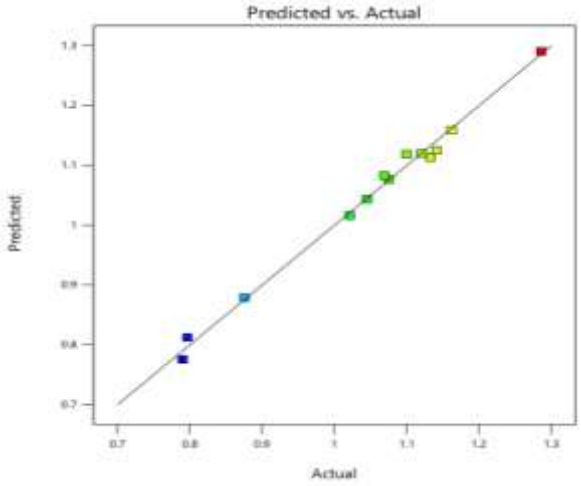
Maximum experimental value of bacterial OD (600 nm) for calcium chloride was 1.29; whereas the corresponding predicted value was 1.21 which obtained in run number (9) using urea 20 g/l (A), calcium chloride 20 g/l (B), and pH 6 (C); however the lowest OD was 0.79 (predicted value was 0.77) obtained in run number (10) using urea 30 g/l (A), calcium chloride 15 g/l (B), and pH 8 (C) as cleared **in table (1)**. Maximum experimental value of bacterial OD (600 nm) for calcium nitrate was 0.988 (predicted value was 0.956) obtained in run number (4) using urea 30 g/l (A), calcium nitrate 10 g/l (B), and pH 6 (C); however, the lowest OD was 0.439 (predicted value was 0.444) obtained in run number (13) using urea 20 g/l (A), calcium nitrate 15 g/l (B), and pH 8 (C) as cleared **in table (1)**. Maximum experimental value of bacterial OD (600 nm) for calcium acetate was recorded in 1.21 (predicted value was 1.19) obtained in run number (1) using urea 10 g/l (A), calcium acetate 1 g/l (B), and pH 7 (C); however the lowest OD was 0.777 (predicted value was 0.774) obtained in run number (13) using urea 20 g/l (A), calcium acetate 1.5 g/l (B), and pH 8 (C) as cleared **in table (1)**. Maximum experimental value of bacterial OD (600 nm) for calcium citrate was recorded in 0.905 (predicted value was 0.909) obtained in run number (4) using urea 30 g/l (A), calcium citrate 100 M (B), and pH 6 (C); however the lowest OD was 0.462 (predicted value was 0.472) obtained in run number (13) using urea 20 g/l (A), calcium citrate 150 M (B), and pH 8 (C) as cleared **in table (1)**.

Maximum experimental value of urease enzyme for calcium chloride was 0.540; whereas the corresponding predicted value was 0.558 which obtained in run number (9) using urea 20 g/l (A), calcium chloride 20 g/l (B), and pH 6 (C); however the lowest urease activity was 0.232 (predicted value was 0.243) obtained in run number (13) using urea 10 g/l (A), calcium chloride 20 g/l (B), and pH 8 (C) as cleared **in table (2)**. Maximum experimental value of urease enzyme for calcium nitrate was 0.441 (predicted value was 0.418) obtained in run number (4) using urea 30 g/l (A), calcium nitrate 10 g/l (B), and pH 6 (C); however, the lowest urease activity was 0.161 (predicted value was 0.151) obtained in run number (13) using urea 20 g/l (A), calcium nitrate 15 g/l (B), and pH 8 (C) as cleared **in table (2)**. Maximum experimental value of urease

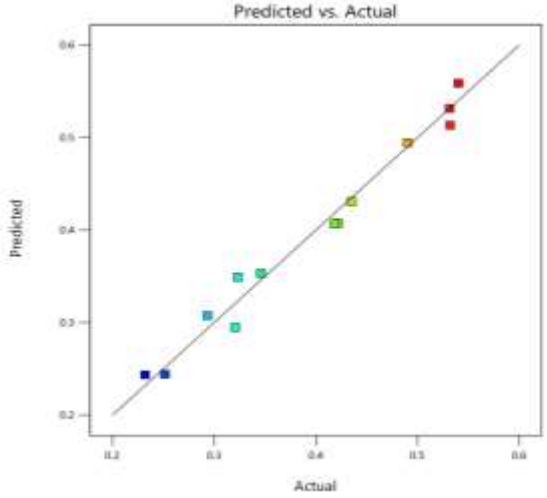
enzyme for calcium acetate was recorded in 0.435 (predicted value was 0.430) obtained in run number (12) using urea 10 g/l (A), calcium acetate 1.5 g/l (B), and pH 6 (C); however, the lowest urease activity was 0.190 (predicted value was 0.169) obtained in run number (6) using urea 10 g/l (A), calcium acetate 1 g/l (B), and pH 8 (C) as cleared **in table (2)**. Maximum experimental value of urease enzyme for calcium citrate was recorded in 0.653 (predicted value was 0.666) obtained in run number (10) using urea 30 g/l (A), calcium citrate 100 M (B), and pH 8 (C); however, the lowest urease activity was 0.281 (predicted value was 0.305) obtained in run number (1) using urea 10 g/l (A), calcium citrate 50 M (B), and pH 7 (C) as cleared **in table (2)**. The predicted values of bacterial OD (600) and urease enzyme (specific activity) were remarked in closely to the response values of the model which indicated the accuracy of the designed model as cleared in **Tables (1&2)** and **figures 1 (a-h)**.

For evaluation the model accuracy, and fitting, evaluating statistical parameter coefficient (R^2) was calculated; R^2 values of OD (600nm) were 0.993 (adjusted R^2 value 0.973) for calcium chloride; 0.987 (adjusted R^2 value 0.951) for calcium nitrate; 0.981 (adjusted R^2 value 0.929) for calcium acetate; 0.992 (adjusted R^2 value 0.97) for calcium citrate. R^2 values of urease activity were 0.979 (adjusted R^2 value 0.919) for calcium chloride; 0.981 (adjusted R^2 value 0.924) for calcium nitrate; 0.973 (adjusted R^2 value 0.894) for calcium acetate; 0.981 (adjusted R^2 value 0.921) for calcium citrate which indicated that the whole variations were explained highly and accurately by the statistical model as cleared in **figures 1 (a-h).**

(a)


Predicted vs. Actual
Predicted
Actual

(b)


Predicted vs. Actual
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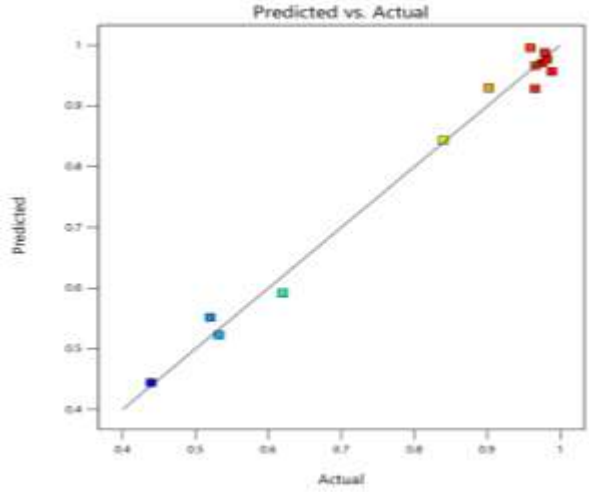
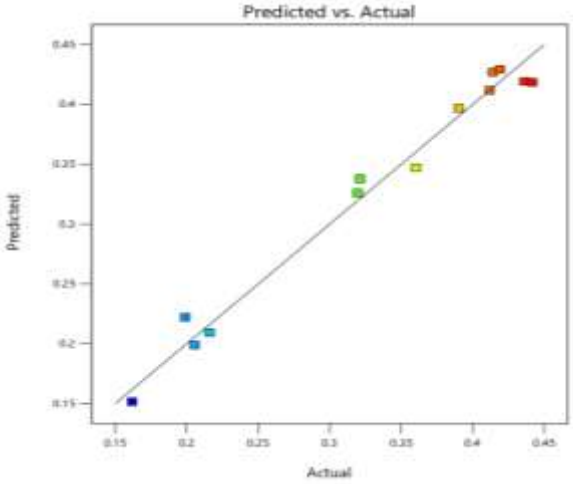
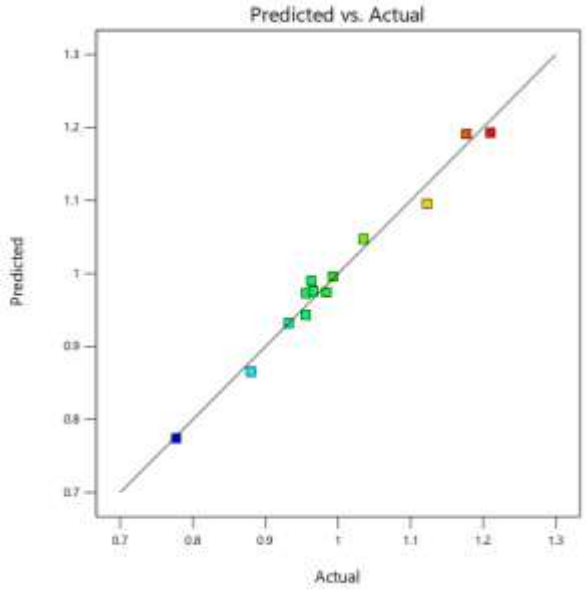
(c)


Predicted vs. Actual
Predicted
Actual

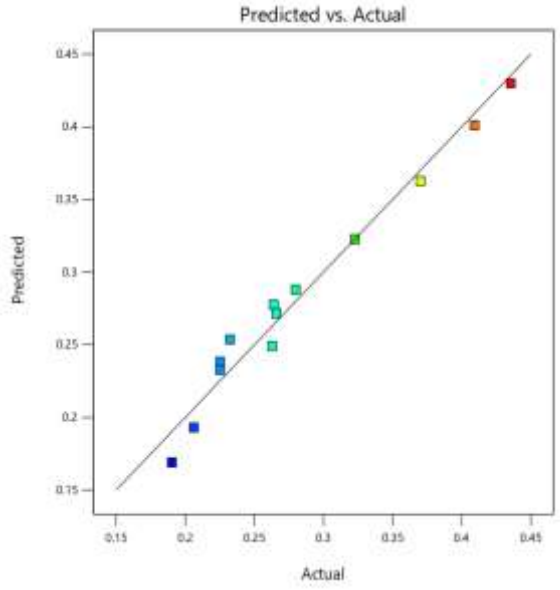
(d)


Predicted vs. Actual
Predicted
Actual

(e)


Predicted vs. Actual
Predicted
Actual

(f)


Predicted vs. Actual
Predicted
Actual

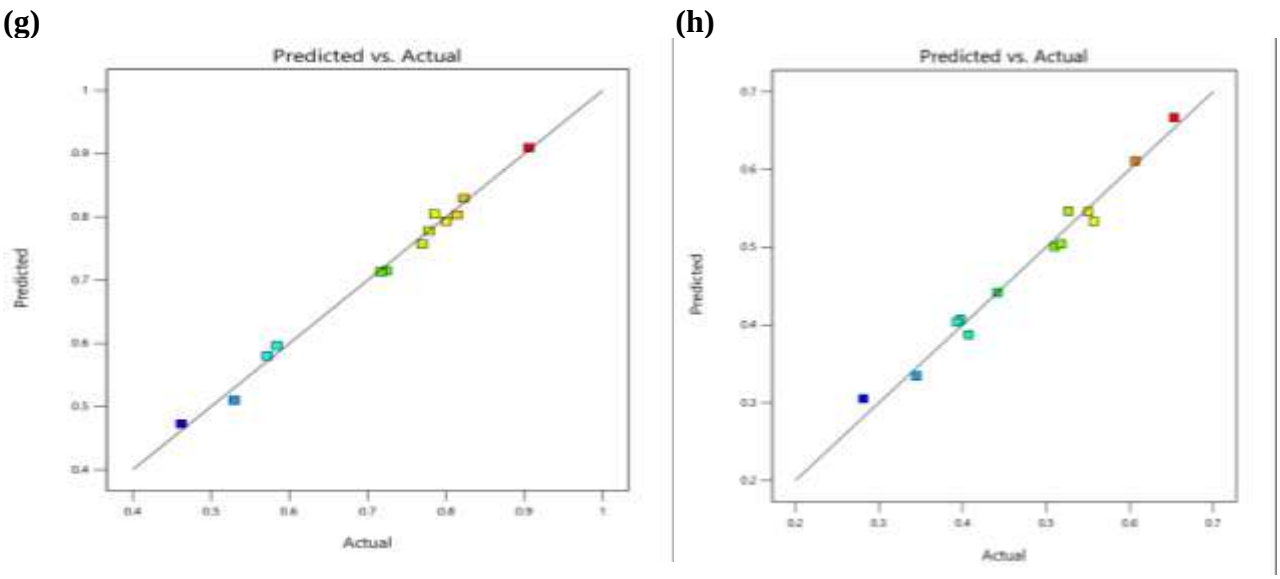


Figure 1(a-h): Comparison between the actual and predicted values of tested design for *Bacillus subtilis* OD (600 nm), a (calcium chloride), b (calcium nitrate), c (calcium acetate), d (calcium citrate) and urease activity e (calcium chloride), f (calcium nitrate), g (calcium acetate), h (calcium citrate) using four calcium salts.

The quadric polynomial equations were further tested for the confirmation of the statistical model suitability, accuracy, and significance via statistical analysis using one way analysis of variance (ANOVA) as cleared in **tables 3&4.** The *F* and *P*- values of the tested model of OD (600 nm) were calculated for calcium chloride (*F;* 48.6, and *P*; 0.0043), calcium nitrate (*F;* 26.85, and *P*; 0.0103), calcium acetate (*F;* 18.48, and *P*; 0.0176), and calcium citrate (*F;* 44.32, and *P*; 0.0049). For urease activity the *F* and *P*- values of the tested model were calculated for calcium chloride (*F;* 16.4, and *P*; 0.0214), calcium nitrate (*F;* 17.23, and *P*; 0.0197), calcium acetate (*F;* 12.27, and *P*; 0.0316), and calcium citrate (*F;* 16.72, and *P*; 0.0204), they all cleared significant models with probability ≤ 0.05) as cleared in **tables 3&4.**

The significance and effects of each individual variable and/ or the interactions were set in **tables (3&4)** as one way ANOVA results. For bacterial OD (600nm), individual variable of (C) pH has significant effects on the OD of the four calcium salts, while urea (A) shows significant ($p<0.005$) effects in calcium acetate and calcium citrate salts and reflects insignificant effect for calcium chloride and calcium nitrate salts. The interaction between different variables AB gives significant ($p<0.005$) interaction for calcium citrates, while interaction AC gives significant interaction for calcium chloride and calcium citrate. The interaction of BC showed

significant ($p<0.005$) results of calcium chloride and calcium acetate. For urease activity, individual variable of (C) pH has significant effects on the OD of all calcium salts except calcium citrate, while urea (A) shows significant ($p<0.005$) effects in calcium citrate salts and reflects insignificant effect for calcium chloride, calcium nitrate, and calcium acetate salts. The interaction between different variables AB gives significant ($p<0.005$) interaction for calcium chloride, while interaction AC gives significant interaction for calcium acetate and calcium citrate. The interaction of BC showed significant ($p<0.005$) results of calcium chloride and calcium citrate as cleared in **tables (3&4)**.

Response surface plots and the contour plots draw for the 3D visualization of the cleared interaction between pair-wise of the two factors when the other factor constant as showed in **figures (2-5)** for urease activity explaining the effect of urea (A), calcium salts (B), and pH (C), on OD (600nm) and urease activity and reflect the interaction between AB (Urea (g/l) * Calcium salts (g/l, M)), AC (Urea (g/l) * pH)), and BC (Calcium salts (g/l, M) * pH) by *Bacillus subtilis*. The previous results showed the effects of three variables (urea (A), calcium salts (B), and pH (C) on OD (600 nm), precipitate (g/l), and urease production by *Bacillus subtilis* bacteria through 13 different runs; however, it is necessary to calculate the most desirable concentrations of the variables from the three tested values. An optima variables concentration for high OD (600 nm), precipitate (g/l), and urease production was estimated from Derringer's desirability function. By applying the function, the obtained optimum levels of OD (600 nm), precipitate (g/l), urease and nitrogenase production by *Bacillus subtilis* gives desirability near to 1.000 **(Figures 6-9).** Also, for model validation, three experiments of the optimum parameters were performed and the mean values were confronted with predicted values. The actual values were in harmony with the predicted once by application the desirability functions which reflect the sufficiency of the quadratic model developed for enhancing the production.

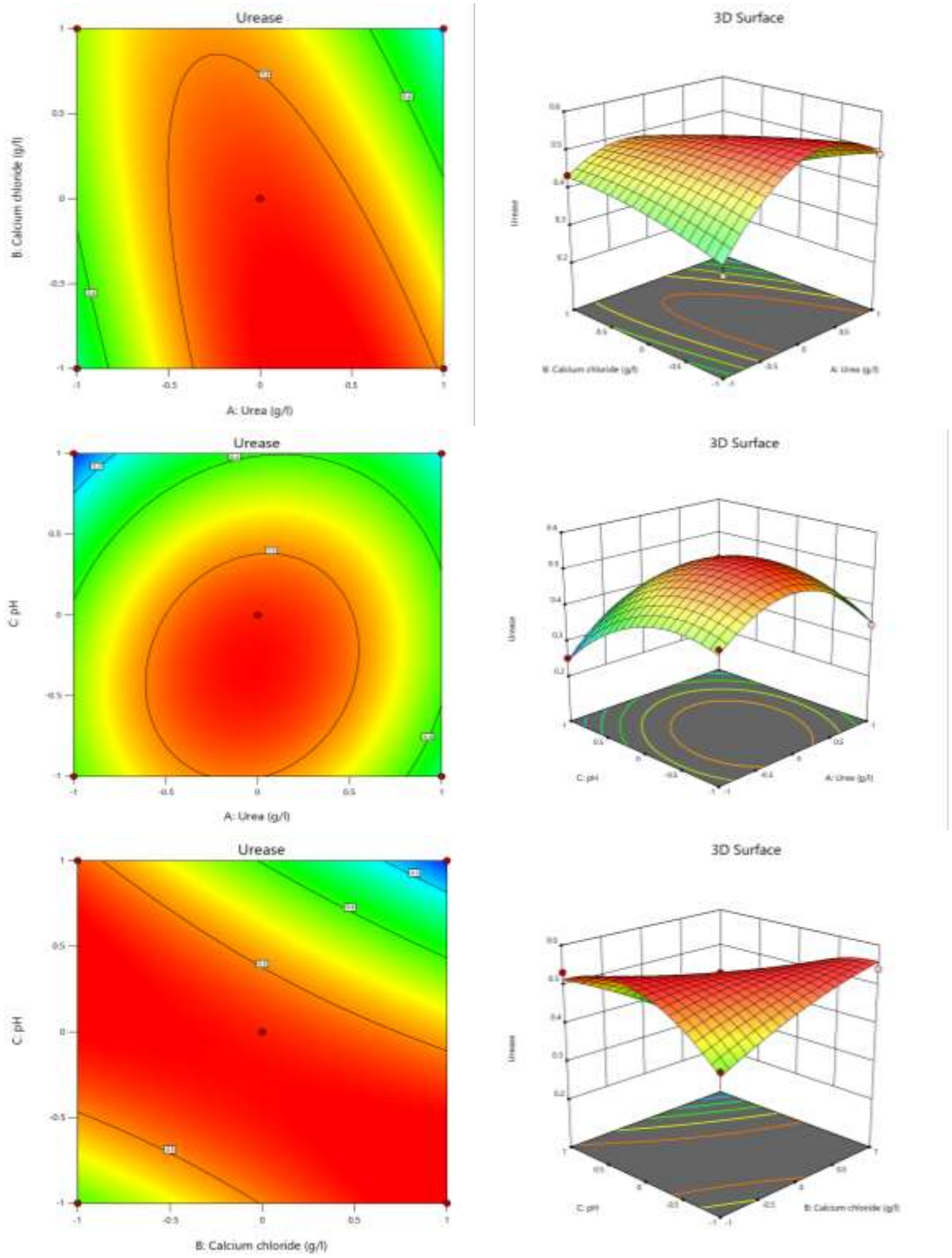


Figure 2: 3D surface plots and contor plots of Box–Behnken statistical experimental design explaining the interactions between AB (urea (g/l) * calcium chloride (g/l)), AC (urea (g/l) * pH), and BC (calcium chloride (g/l) * pH) on urease by *Bacillus subtilis*.

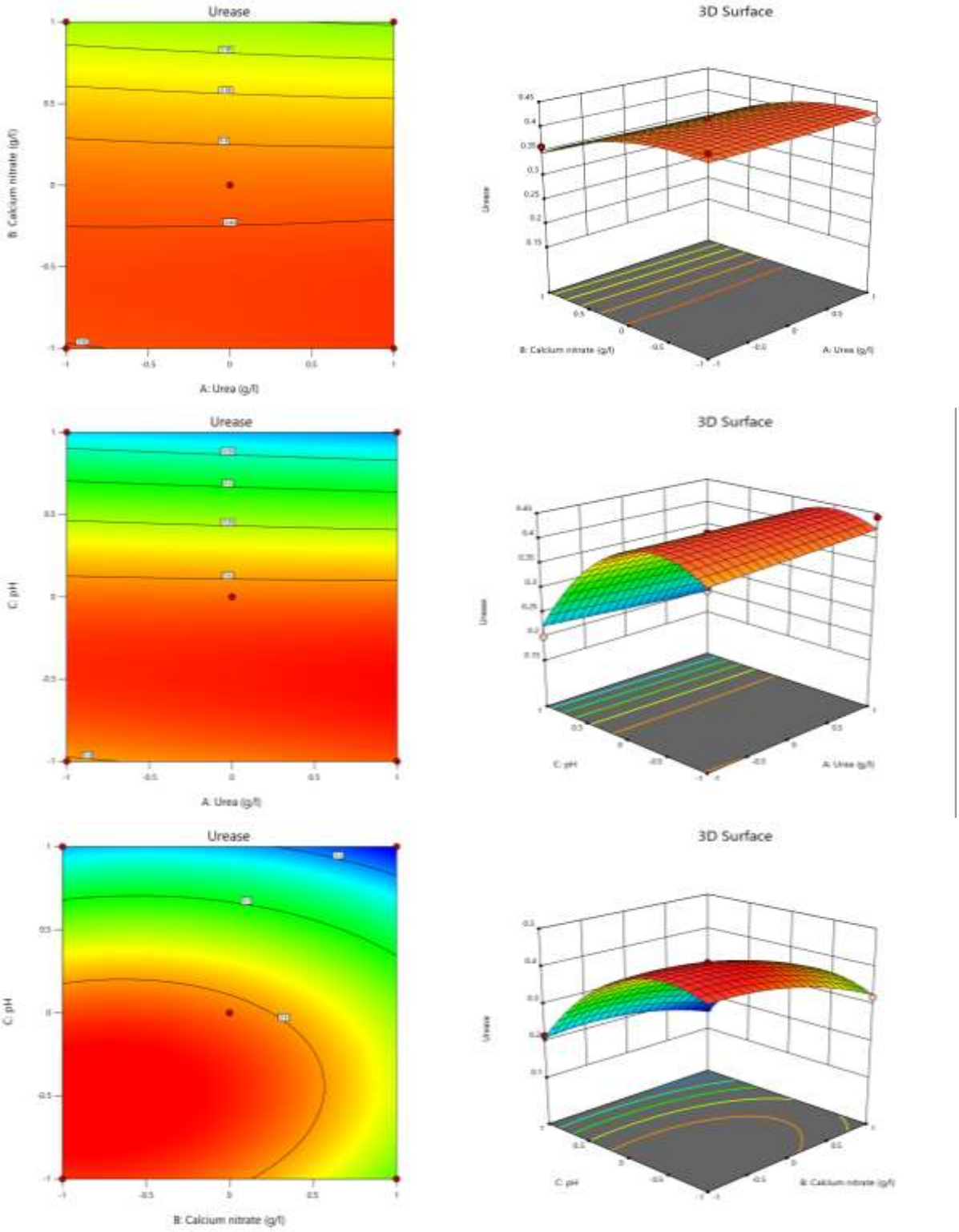


Figure 3: 3D surface plots and contor plots of Box–Behnken statistical experimental design explaining the interactions between AB (urea (g/l) * calcium nitrate (g/l)), AC (urea (g/l) * pH), and BC (calcium nitrate (g/l) * pH) on urease by *Bacillus subtilis*.

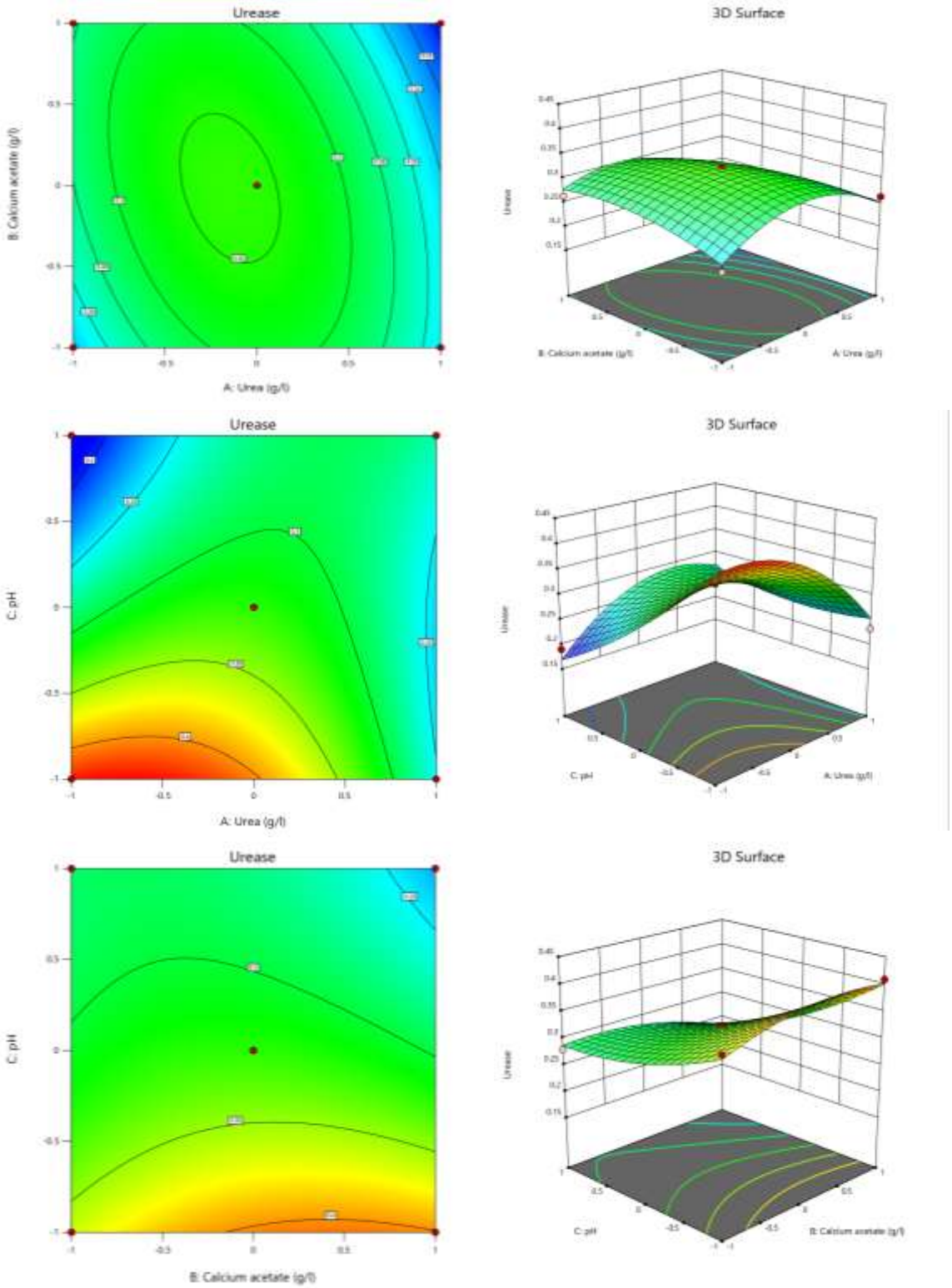


Figure 4: 3D surface plots and contor plots of Box–Behnken statistical experimental design explaining the interactions between AB (urea (g/l) * calcium acetate (g/l)), AC (urea (g/l) * pH), and BC (calcium acetate (g/l) * pH) on urease by *Bacillus subtilis*.

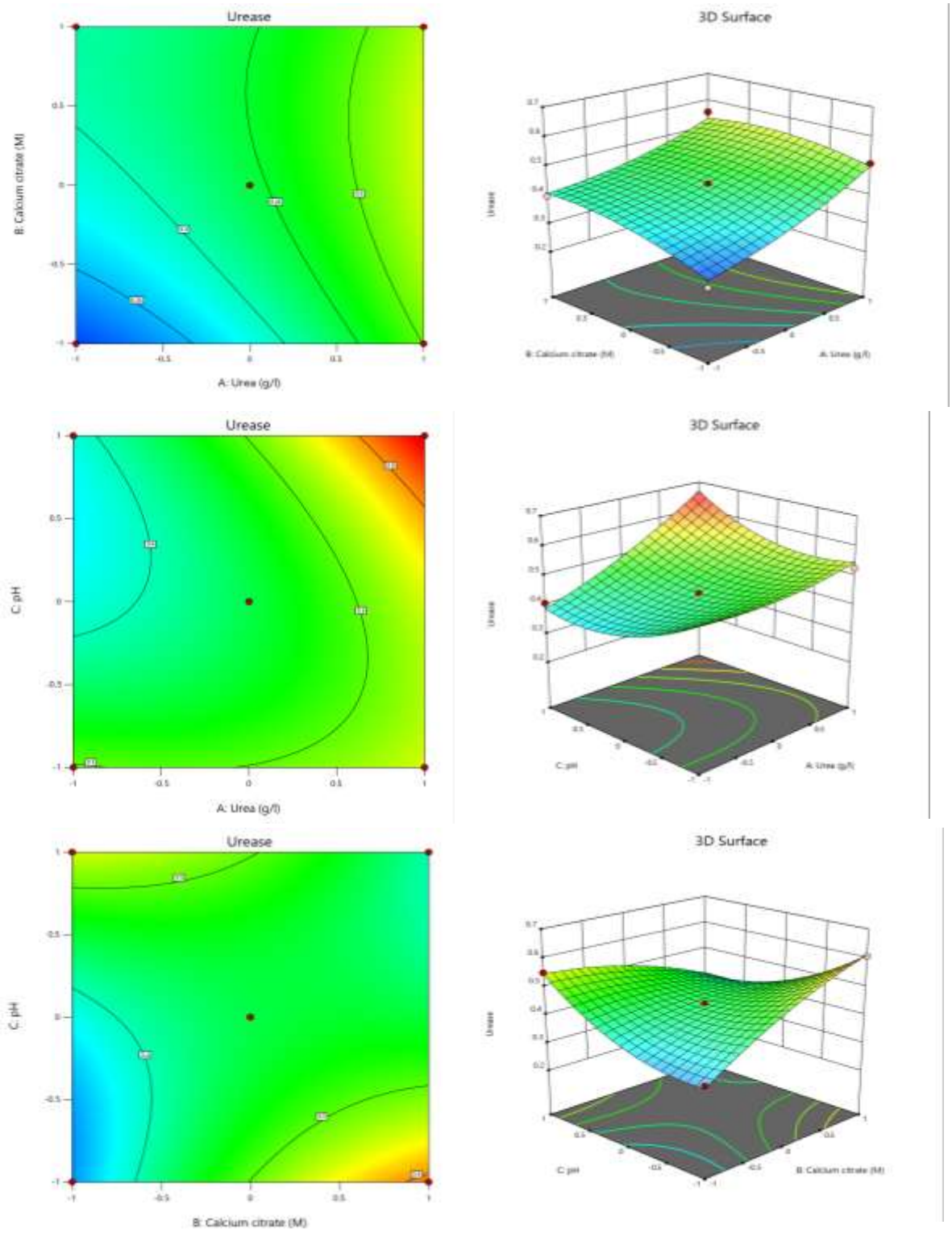


Figure 5: 3D surface plots and contor plots of Box–Behnken statistical experimental design explaining the interactions between AB (urea (g/l) * calcium citrate (g/l)), AC (urea (g/l) * pH), and BC (calcium citrate (g/l) * pH) on urease by *Bacillus subtilis*.

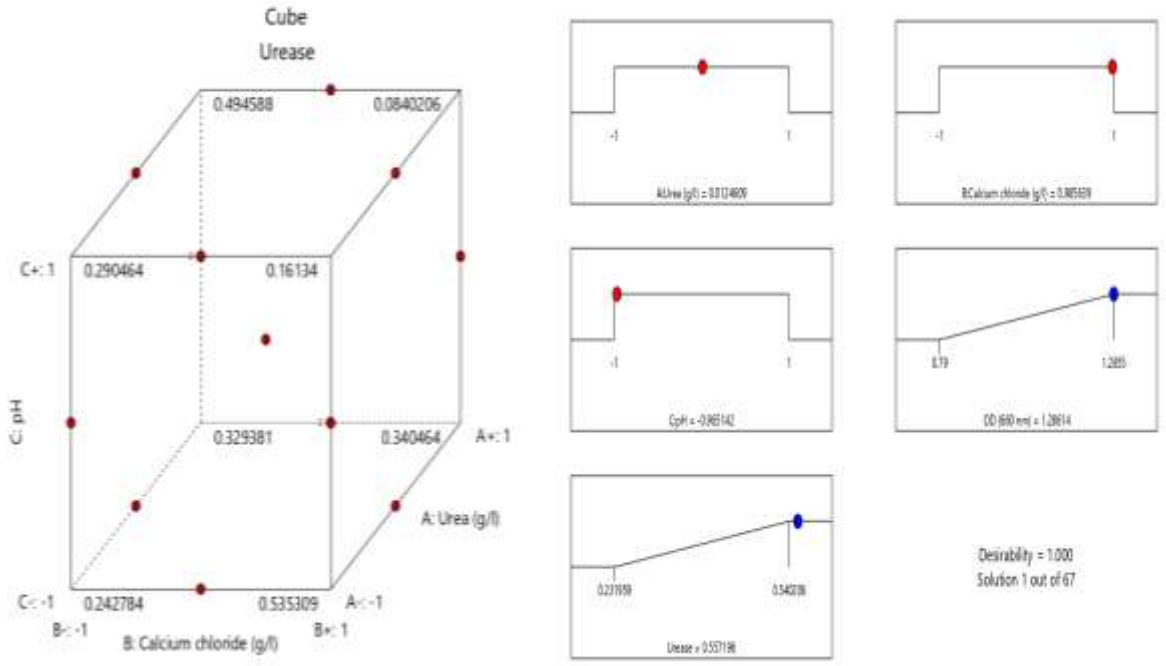


Figure 6: The statistical optimization desirability ramp plot for urease activity by *Bacillus subtilis* using calcium chloride salt.

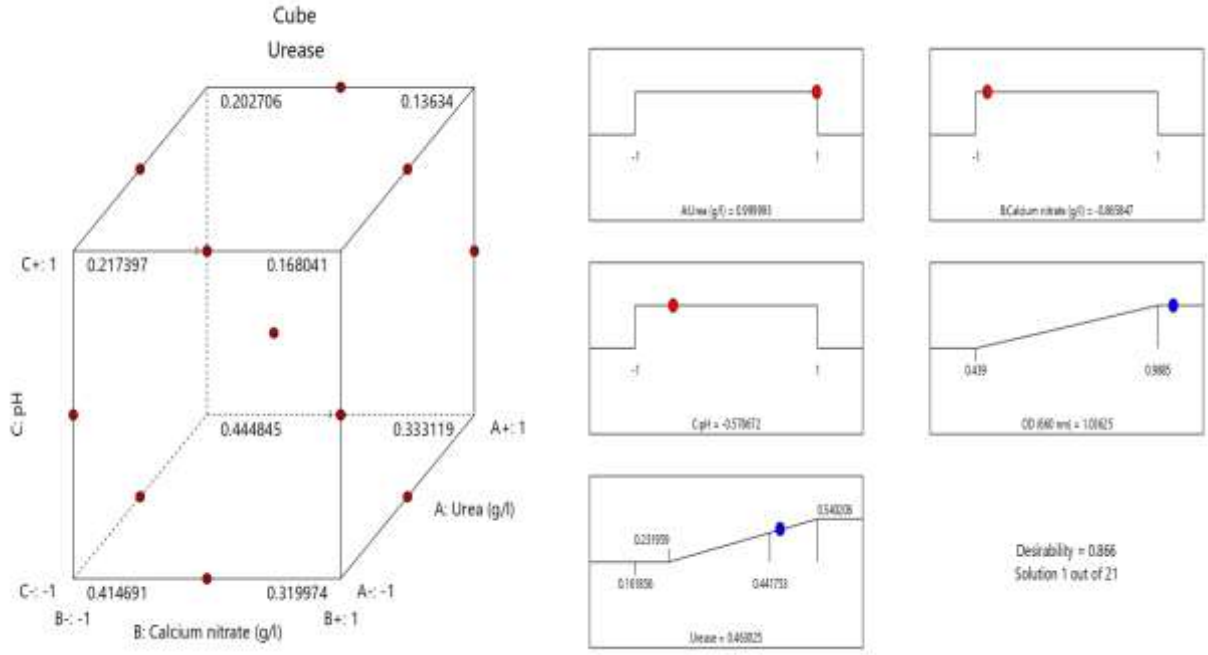


Figure 7: The statistical optimization desirability ramp plot for urease activity by *Bacillus subtilis* using calcium nitrate salt.

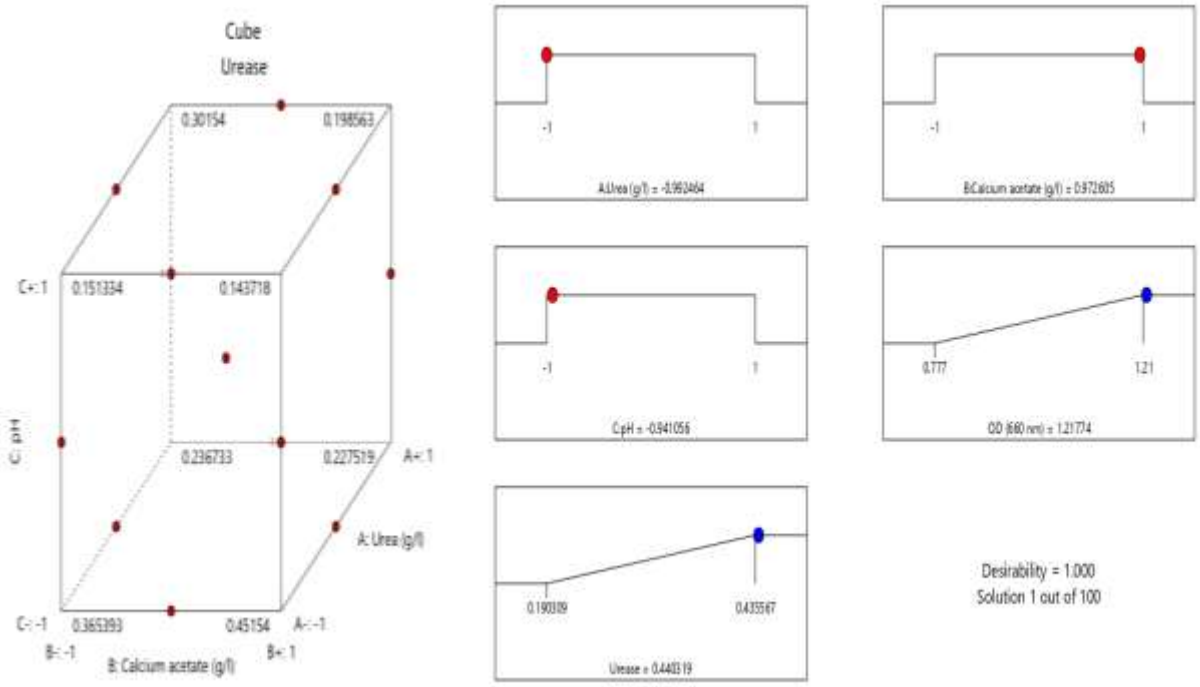


Figure 8: The statistical optimization desirability ramp plot for urease activity by *Bacillus subtilis* using calcium acetate salt.

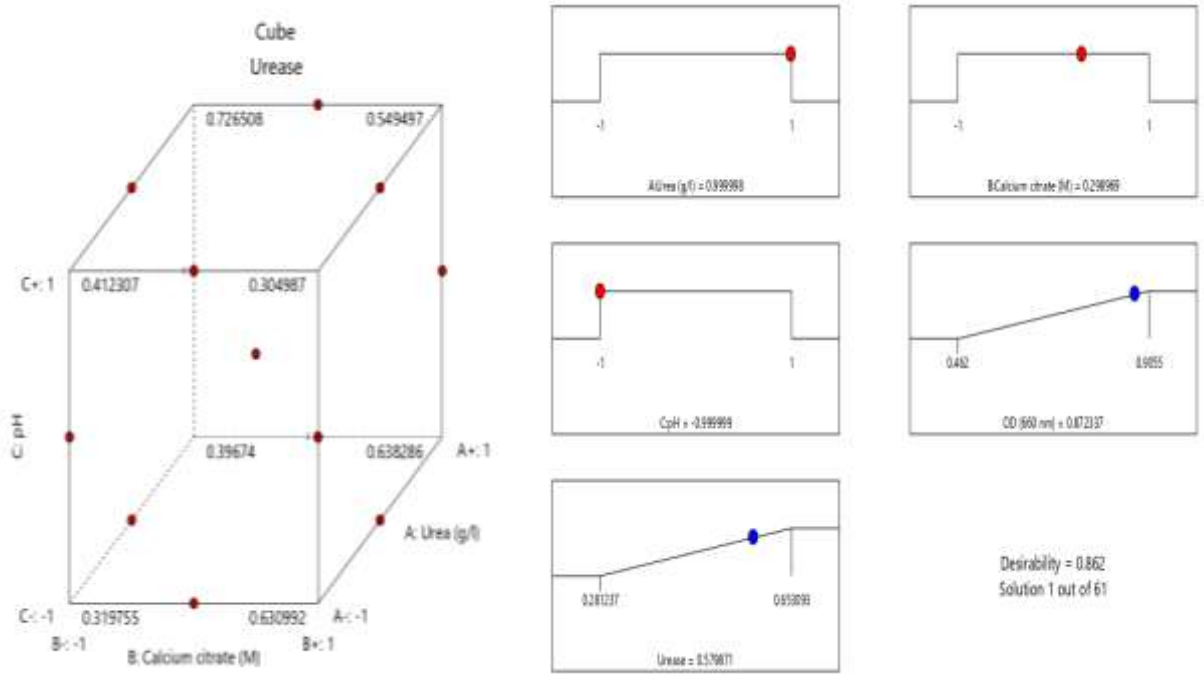


Figure 9: The statistical optimization desirability ramp plot for urease activity by *Bacillus subtilis* using calcium citrate salt.

Table 1: Box-Behnken design with three variables; urea (g/l) (A), calcium salts (B), and pH (C), with actual and predicted responses of *Bacillus subtilis* OD (600 nm) using four calcium salts

Trial	Urea (g/l)	Calcium salts	pH	Calcium chloride		Calcium nitrate		Calcium acetate		Calcium citrate	
				Actual values	Predict values	Actual values	Predict values	Actual values	Predict values	Actual values	Predict values
1	-1	-1	0	1.07	1.08	0.975	0.971	1.21	1.19	0.723	0.714
2	-1	1	0	1.13	1.11	0.965	0.928	1.03	1.05	0.800	0.793
3	0	-1	-1	1.14	1.13	0.982	0.976	0.993	0.995	0.814	0.803
4	1	0	-1	1.16	1.16	0.988	0.956	0.966	0.975	0.905	0.909
5	0	0	0	1.12	1.12	0.966	0.966	0.932	0.932	0.778	0.778
6	-1	0	1	0.875	0.889	0.520	0.551	0.984	0.974	0.717	0.713
7	1	1	0	1.04	1.04	0.839	0.843	0.956	0.973	0.571	0.580
8	0	-1	1	1.02	1.02	0.619	0.591	0.963	0.989	0.584	0.596
9	0	1	-1	1.29	1.21	0.902	0.929	1.12	1.10	0.769	0.756
10	1	0	1	0.79	0.77	0.532	0.522	0.880	0.865	0.529	0.510
11	1	-1	0	1.10	1.12	0.959	0.995	0.955	0.943	0.822	0.829
12	-1	0	-1	1.07	1.08	0.979	0.988	1.18	1.19	0.785	0.804
13	0	1	1	0.796	0.812	0.439	0.444	0.777	0.774	0.462	0.472

Table 2: Box-Behnken design with three variables; urea (g/l) (A), calcium salts (B), and pH (C), with actual and predicted responses of *Bacillus subtilis* urease enzyme (specific activity) using four calcium salts

Trial	Urea (g/l)	Calcium salts	pH	Calcium chloride		Calcium nitrate		Calcium acetate		Calcium citrate	
				Actual values	Predict values	Actual values	Predict values	Actual values	Predict values	Actual values	Predict values
1	-1	-1	0	0.323	0.349	0.436	0.419	0.225	0.238	0.281	0.305
2	-1	1	0	0.435	0.430	0.360	0.347	0.263	0.277	0.397	0.407
3	0	-1	-1	0.418	0.407	0.419	0.429	0.370	0.362	0.344	0.334
4	1	0	-1	0.345	0.353	0.441	0.418	0.232	0.253	0.526	0.546
5	0	0	0	0.531	0.531	0.411	0.411	0.322	0.322	0.441	0.441
6	-1	0	1	0.251	0.244	0.199	0.222	0.190	0.169	0.407	0.387
7	1	1	0	0.320	0.294	0.321	0.338	0.206	0.193	0.557	0.533
8	0	-1	1	0.532	0.513	0.216	0.209	0.279	0.288	0.550	0.545
9	0	1	-1	0.540	0.558	0.319	0.326	0.409	0.401	0.606	0.611
10	1	0	1	0.293	0.307	0.205	0.199	0.266	0.271	0.653	0.666
11	1	-1	0	0.490	0.494	0.413	0.427	0.262	0.249	0.510	0.500
12	-1	0	-1	0.421	0.407	0.390	0.396	0.435	0.430	0.517	0.504
13	0	1	1	0.232	0.243	0.161	0.151	0.225	0.232	0.393	0.403

Table 3: ANOVA results for Box-Behnken quadratic model of *Bacillus subtilis* OD (600 nm) using four calcium salts

Source	Calcium chloride			Calcium nitrate			Calcium acetate			Calcium citrate		
	Mean Square	***F*-value**	***p*-value**	**Mean Square**	***F*-value**	***p*-value**	**Mean Square**	***F*-value**	***p*-value**	**Mean Square**	***F*-value**	***p*-value**
Model	0.0281	48.61	0.0043	0.0583	26.85	0.0103	0.0182	18.48	0.0176	0.0235	44.32	0.0049
A-Urea (g/l)	0.0004	0.6545	0.4777	0.0018	0.8357	0.4280	0.0525	53.30	0.0053	0.0048	9.11	0.0568
B-Calcium salts	0.0007	1.28	0.3397	0.0190	8.73	0.0598	0.0067	6.77	0.0802	0.0145	27.44	0.0135
C-pH	0.1723	298.20	0.0004	0.3789	174.45	0.0009	0.0535	54.29	0.0052	0.1204	227.34	0.0006
AB	0.0031	5.43	0.1022	0.0030	1.39	0.3230	0.0077	7.81	0.0681	0.0268	50.62	0.0057
AC	0.0080	13.86	0.0337	0.5630	0.07	0.9803	0.0028	2.89	0.1878	0.0237	44.77	0.0068
BC	0.0340	58.92	0.0046	0.0025	1.16	0.3599	0.0249	25.26	0.0152	0.0015	2.83	0.1908
A^2	0.0081	14.01	0.0333	0.0001	0.0411	0.8523	0.0120	12.18	0.0398	0.0005	0.8610	0.4219
B^2	0.0017	2.99	0.1821	0.0015	0.6843	0.4688	0.0027	2.79	0.1936	0.0090	16.99	0.0259
C^2	0.0171	29.60	0.0122	0.0964	44.39	0.0069	0.0000	0.0180	0.9017	0.0077	14.45	0.0320

Table 4: ANOVA results for Box-Behnken quadratic model of *Bacillus subtilis* urease enzyme (specific activity) using four calcium salts

Source	Calcium chloride			Calcium nitrate			Calcium acetate			Calcium citrate		
	Mean Square	***F*-value**	***p*-value**	**Mean Square**	***F*-value**	***p*-value**	**Mean Square**	***F*-value**	***p*-value**	**Mean Square**	***F*-value**	***p*-value**
Model	0.0153	16.14	0.0214	0.0135	17.13	0.0197	0.0079	12.27	0.0316	0.0153	16.72	0.0204
A-Urea (g/l)	0.0000	0.0453	0.8452	0.0196	0.0015	0.9714	0.0027	4.22	0.1321	0.0517	56.66	0.0049
B-Calcium salts	0.0070	7.33	0.0734	0.0130	16.43	0.0270	0.0001	0.2196	0.6713	0.0090	9.88	0.0516
C-pH	0.0218	22.91	0.0173	0.0776	98.36	0.0022	0.0295	45.78	0.0066	0.080	0.0078	0.9353
AB	0.0198	20.82	0.0197	0.0001	0.0916	0.7819	0.0023	3.52	0.1571	0.0012	1.33	0.3322
AC	0.0035	3.63	0.1528	0.0005	0.6369	0.4832	0.0194	30.14	0.0119	0.0141	15.42	0.0294
BC	0.0444	46.74	0.0064	0.0005	0.6516	0.4786	0.0022	3.41	0.1621	0.0438	48.02	0.0062
A^2	0.0334	35.12	0.0096	0.0673	0.0008	0.9796	0.0087	13.45	0.0351	0.0013	1.40	0.3213
B^2	0.0008	0.8049	0.4358	0.0020	2.52	0.2105	0.0011	1.64	0.2903	0.0019	2.08	0.2447
C^2	0.0155	16.35	0.0272	0.0244	30.85	0.0115	0.0009	1.41	0.3198	0.0084	9.26	0.0557

DISCUSSION

Bacterial activity and response rates are two examples of process restrictions that can be identified to control MICP for its use in geotechnical engineering. Real-world MICP use necessitates knowledge of how different treatment modalities may affect particular applications. Despite the lack of quantitative engineering property measurements on post-treatment samples, this study highlights the significance of treatment method to guarantee that the treatment used is not only appropriate for field conditions but also optimal for the application for which it was designed and produces the best results from the process. The precipitation process is controlled chemically and biologically by the presence of calcium and carbon sources, as well as the ability of bacteria to hydrolyze urea and produce alkalinity and carbonate. The rate at which bacteria hydrolyze urea, or bacterial activity, may be a good way to measure this. However, the rate of precipitation in the field is not necessarily determined by it because there are several other factors that could influence the process when it comes to response in porous media.

Recently statistical experimental designs were utilized highly in the optimization process over the traditional methods (one factor and two factors at a time) for saving time, costs, minimizing the experimental errors [13, 14]. These methods showing the interaction between several parameters in the same time and their effects on the final product [15]. Also, the statistical coefficient (R^2) assessed for the statistical designs helps to clear the model goodness, accuracy, and fitting [16]. In our design, the accuracy of the design could clearly be observed from the statistical coefficient (R^2) 0.979 (calcium chloride), 0.981 (calcium nitrate), 0.973 (calcium acetate), and 0.981 (calcium citrate) indicating that all set variations were illustrated significantly via the whole statistical model.

Using urea 20 g/l (A), calcium chloride 20 g/l (B), and pH 6 (C), this study demonstrated that the maximum specific activity of the urease enzyme for calcium chloride was 0.540 (predicted value 0.558) obtained in run (9) whereas calcium nitrate was 0.441 (predicted value 0.418) obtained in run (4) using urea 30 g/l (A), calcium nitrate 10 g/l (B), and pH 6 (C). In run number (12), calcium acetate was measured at

0.435 (predicted value 0.430) using urea 10 g/l (A), calcium acetate 1.5 g/l (B), and pH 6 (C); in run number (10) calcium citrate was measured at 0.653 (predicted value 0.666) using urea 30 g/l (A), calcium citrate 100 M (B), and pH 8 (C).

The ureolytic bacterial strains were recently discovered by Dhami [17] from calcareous soil samples that were taken in Andhra Pradesh, India. Five positive strains (*B. megaterium, B. thuringiensis, B. cereus, B. subtilis, and L. fusiformis*) were selected based on urease activity and calcite precipitation. However, *B. megaterium* exhibited the highest urease activity (690 U/ml) and calcite precipitation (187 mg/100 ml) [18]. Urease activity and calcite production were shown by four additional urease-producing bacteria that were isolated from abandoned mining sites and an abandoned expressway in Gangwondo, Korea [19]. Only two *Lysinibacillus sp*. strains, nevertheless, were able to generate significant urease activity and calcite precipitation. From abandoned expressway sites in Gangwondo, Korea, the same group isolated another strain of urease-producing bacteria, *Sporosarcina pasteurii* WJ-2 (Unpublished data). After screening three distinct bacteria for urease production, [20] discovered that *B. pasteurii* was able to produce urease at a level that was around two times higher than that of the other examined microbes. Additionally, ureolytic activity has been found in situ in natural soil and ground water systems by several researchers [21,22,23].

Because the urease enzyme is only active at pH values specific to urea hydrolysis, calcite precipitation is regulated by pH. According to numerous researchers, urease's ideal pH is 8.0, beyond which the enzyme's activity declines [5,24]. For urea hydrolysis to produce ammonia, a high pH is necessary. Aerobic bacteria produce ammonia, which raises pH and causes CO_2 to be released by cell respiration [25]. The carbonate will typically dissolve rather than precipitate if the pH drops (Loewenthal and Marais 1978). The alkaline pH range of 8.7 to 9.5 is where the majority of calcite precipitation occurs [5,26,27]. However, [28] discovered that the ideal pH and acid urease were almost neutral. Recently, [29] looked into whether halophilic and alkaliphilic urease-producing bacteria are active in environments that are suitable for creating biocement, such as high inorganic salt concentrations and pH values above 8.5[30, 31].

CONCLUSION

This is study showed the maximum value of urease production from the microbial strains is considered the first step for efficient utilization of the product. *Bacillus subtilis* OQ119616 was isolated from Sahel Salim soil sample and identified phenotypic and genotypic using 16SrRNA gene sequence. Box–Behnken statistical experimental design with 13- runs were utilized to optimize and evaluate the interaction effects, main effects, and the quadratic effects of four different Ca-salts on the OD (600 nm) and urease specific activity by *B. subtilis* in liquid media. the maximum specific activity of urease enzyme for calcium chloride was 0.540 (predicted value 0.558) obtained in run (9) using urea 20 g/l (A), calcium chloride 20 g/l (B), and pH 6 (C), while calcium nitrate was 0.441 (predicted value 0.418) obtained in run (4) using urea 30 g/l (A), calcium nitrate 10 g/l (B), and pH 6 (C). For calcium acetate was 0.435 (predicted value 0.430) obtained in run number (12) using urea 10 g/l (A), calcium acetate 1.5 g/l (B), and pH 6 (C), while calcium citrate was 0.653 (predicted value 0.666) obtained in run number (10) using urea 30 g/l (A), calcium citrate 100 M (B), and pH 8 (C). R^2 values of urease specific activities were 0.979 (calcium chloride), 0.981 (calcium nitrate), 0.973 (calcium acetate), and 0.981 (calcium citrate).

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