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Evaluation of stabilized soil by *Bacillus* sp. HAI4 in different conditions through Taguchi method

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Abstract

Introduction: The process of calcite precipitation resulted from metabolic activities by microorganisms is biocalcification. In this process, calcite ($CaCO_3$) is deposited on the soil grains by metabolic activities of microorganisms resulting in increased stiffness/strength and reduced erodibility of soil.

Materials and methods: In this study, urea hydrolysis and calcite precipitation rates in soil stabilization process were evaluated by the bacterium *Bacillus* sp. HAI4 through Taguchi method. Also, it was designed a wind tunnel for analysis of soil stabilization rate.

Results: In this investigation, three factors (NH_4Cl, urea and molasses) were surveyed in optimization of soil stabilization .Results of this study illustrated that Taguchi method is one of effective methods for optimization of effective factors in soil stabilization process. In this case, urea concentration had higher effect on soil stabilization and calcite sedimentation by *Bacillus* sp. Also, evaluating factors interaction showed relationship of factors together. Three levels (0.5, 1 and 1.5 g/L), were determined for NH_4Cl factor in which the highest effect (4.353%) in soil stabilization was related to level 3. Increasing of NH_4Cl resulted in high soil stabilization rate. Three levels 20, 30, and 40 g/L of urea were applied. Results illustrated that effective level was 3 with 4.313%. Also, Taguchi design was in this study, as three levels of molasses (30, 40 and 50 gr/L) illustrated maximum soil stabilization in level 2 by 3.873%. In fact, by increasing of molasses, soil stabilization firstly increased and then decreased.

Discussion and conclusion: Totally, this study shows two factors (NH_4Cl and urea) that have a major effect in urea hydrolysis and calcite precipitation rates.

Key words: Biocalcification, Soil stabilization, *Bacillus* sp., Urea hydrolysis, Calcite precipitation, Ammonium chloride

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Introduction

Biocalcification has illustrated some promise in soil cementation by microbially induced calcite precipitation (1, 2). In this process, calcite ($CaCO_3$) is deposited on the soil grains by metabolic activities of microorganisms resulting in increased stiffness/strength and reduced erodibility of soil. In this case, urea hydrolysis by some bacteria such as *Bacillus pasteurii* or *sporosarcina pasteurii* can lead to calcite precipitation as crystals (3-5).

Using this method has illustrated a promise in several fields such as strengthening of concrete and cracks remediation (6, 7), remediation of copper-contaminated soil (8), improving of strength and durability of fly ash-amended concrete (9), Biomineralization based remediation of As(III) contaminated soil (10, 11), Bioremediation of Pb-contaminated soil (12), improvement in the durability of cement mortar (13) and improving of the stiffness/strength of sandy soil (14).

Biomineralization as production of minerals by living organisms (14) is resulted from metabolic activities of some organisms including invertebrates (carbonate), vertebrates (carbonates, phosphates and calcium), algae and diatoms (silicate) (14, 15). In the case of biologically induced mineralization (BIM), the minerals are produced extracellular by metabolic activities of the living organisms (16).

Four important factors which affect calcite precipitation include 1) concentration of the calcium, 2) dissolved inorganic carbon (DIC) concentration, 3) the pH and (4) the availability of nucleation sites (17-19). Different bacterial species can precipitate carbonates in environment with high pH and Ca^{2+} ions. In addition, various mechanisms can stimulate this process by bacteria (17, 20). Ability of bacteria to create environment with alkaline condition are important mechanisms. Carbonate precipitation can be developed as extracellular by successive stratification and it leads to growing carbonate crystals on the bacteria surface (21-23).

In order to calcium carbonate production, several studies applied urea and $CaCl_2$ combinations. For example, different combinations of 0.33 and 0.67 M urea and 0.0025, 0.025 and 0.25M Ca^{2+} solution were used by Okwada and coworkers (2010). The results of this study, illustrate that the rate of ureolysis increases with bacterial cell concentration, and the bacterial cell concentration had a greater influence on k(urea) than initial urea concentration (24). Also, application of lower chemical amounts used over multiple injections resulted in more uniform cementation (25). In this study, urea hydrolysis and calcite precipitation rates in soil stabilization process were evaluated for the bacterium *Bacillus* sp. HAI4 by Taguchi method in three levels of three factors (NH_4Cl, urea and molasses).

Materials and methods

Particle size analysis of soil: Sufficient aggregated samples were obtained from Wetland Hooralazim of Khuzestan province in Iran. In order to analyze soil particle size, a gradation test was performed on a soil sample in a laboratory(26). In this case, a determined weighed sample was poured into the top sieve which had a set of sieves with progressively smaller openings (Fig. 1). A mechanical shaker was used for shaking of sieve column. The results were demonstrated in a graph of percent passing versus the diameter of sieves (4.75, 2.36, 2, 1.4, 1.18, 0.85, 0.71, 0.6, 0.425, 0.3, 0.25, 0.15, 0.075) (Table 1).

Fig. 1- Used columns of laboratory sieve in this study

Table 1- Sieve analysis results are generally expressed as the percentage of the total weight of soil that passed through different sieves

Sieve number	Diameter (mm)	Mass of soil retained on each sieve (g)	Percent retained (%)	Cumulative passed (%)
4	4.75	18.53	18.53	81.47
8	2.36	11.9	30.43	69.57
10	2	2.87	33.3	66.7
14	1.4	8.51	41.81	58.19
16	1.118	3.45	45.26	54.74
20	0.85	4.3	49.56	50.44
25	0.71	5.44	55	45
30	0.6	0.178	55.178	44.822
40	0.425	7.866	63.044	36.956
50	0.3	4.58	67.624	32.376
60	0.25	1.54	69.164	30.836
100	0.15	9.36	78.524	21.476
200	0.075	8.018	86.542	13.458
pan	pan	13.458	100	0
		100		

Bacteria preparation and treatment: *Bacillus* sp. HAI4 species were isolated form from Wetland Hooralazim of Khuzestan province in Iran. Species were grown in broth nutrient at 30C for 24 hours in incubator. In order to bacterial calcite precipitation, nutrient broth-urea containing 30 g urea, 30 g molasses, 5 g NH_4Cl and 5 g milk powder per liter (10pH) was autoclaved, cooled and combined immediately with desired concentration of filtered (0.2 Mm) and sterilized $CaCl_2$ before application onto soil. Culture medium was incubated at 30 ˚C for 48 hours. After time incubation, prepared bacteria were colored by methylene blue.

Optimization of soil stabilization by Bacteria: For obtain optimum condition of soil stabilization by bacteria, Taguchi method was applied in three levels for three factors (NH_4Cl, urea and molasses) (Table 2). In this case, nine experiments were designed (Table 3). Milk powder concentration (0.1 g/L) was fixed for each experiment.

Table 2- Design of three factors with three levels by Taguchi method

Factors (g/L)	Level 1	Level 2	Level 3
NH_4Cl	0.5	1	1.5
Urea	20	30	40
Molasses	30	40	50

Table 3- Nine experiments were designed by Taguchi method

Experiment number	NH_4Cl (Level):(g/L)	Urea (Level):(g/L)	Molasses (Level):(g/L)
1	1= 0.5	1= 20	1= 30
2	1= 0.5	2= 30	2= 40
3	1= 0.5	3= 40	3= 50
4	2= 1	1= 20	2= 40
5	2= 1	2= 30	3= 50
6	2= 1	3= 40	1= 30
7	3= 1.5	1= 20	3= 50
8	3= 1.5	2= 30	1= 30
9	3= 1.5	3= 40	2= 40

In addition to nine designed experiments, two experiments were designed as control (Table 4). One experiment, number ten, contains treatment solution without bacteria and other experiment contains only bacteria without treatment solution.

Table 4- Control experiment designed by Taguchi Method

Experiment number	NH_4Cl (g/L)	Urea (Level):(g/L)	Molasses (Level):(g/L)
10	2= 1	2= 30	2= 40

On the first day, eleven flasks contain 100 g of soil were prepared and 70 ml of bacteria with 0.5 McFarland was added to all flaks apart from control flask with number 10. Afterwards, 100 ml of treatment solutions were prepared and since the second day, 20ml of solutions were added to soils apart from controls for five days. Every day, after adding solutions, soils were incubated at 30C for 24 hours.

Biological cementation: Treatment solutions (100 mm) were prepared with adding 2.2g calcium chloride ($CaCl_2$). Afterwards, 20 mm of treatment solution were added to each soil for 5 days (apart from control without bacteria). Due to complete drying, after five days, all soils were incubated for one mount at 30 C.

Wind tunnel testing: To analyze soil fixation, wind velocity was measured by design wind tunnel (27). Wind velocity was evaluated by the following formula:

$$v^2 = \frac{mg\ tan\theta}{\frac{1}{2}cpA}$$

Where ν as velocity
$m=7.5\times10^{-3}$ kg (mass of bullet)
$g=9.8$ m/s^2
tanθ as angle of deviation
C as constant of integration equals 0/6
ρ as air density equals 1.3kg/m^3
A as area of bullet.

In this case, two experiments at two different angles (8˚ and 12˚) were designed.

After this stage, each of weighted soil samples was exposed to wind velocity by 14.56 km/h. Then, reduced weight of soil samples was measured through weigh again of samples (Table 5). This process was applied for wind velocity by 18km/h.

$\tan 8^\circ = 0.14$

$$v_1^2 = \frac{7/5\times10^{-3}\times9/8\times\tan8^\circ}{1/2\times0/6\times1/3\times16\times10^{-4}} = 16/55\ {}^{m^2}/_{s^2}$$

$$\Longrightarrow v_1 = 4/1\ {}^{m}/_{s} = 14/56\ {}^{km}/_{h}$$

$\tan 12^\circ = 0/21$

$$v_2^2 = \frac{7/5\times10^{-3}\times9/8\times\tan12^\circ}{1/2\times0/6\times1/3\times16\times10^{-4}} = 25\ {}^{m^2}/_{s^2}$$

$$\Longrightarrow v_2 = 5\ {}^{m}/_{s} = 18\ {}^{km}/_{h}$$

Fig. 2- Applied wind tunnel in this study

Results

Soil fixation: Interaction of factors and optimum conditions in soil stabilization was evaluated by Qualitek-4 software. Soil stabilization rate in 11 experiments illustrates those bacteria in medium by 1g/L ammonium Chloride, 40 g/L urea and 30 g/L molasses, had maximum of soil stabilization in experiment number 6.

Table 6, demonstrates effect of each factors and their levels on soil fixation. In this case, best level is for ammonium chloride at third level (1.5 g/L), Urea at third level (40g/L), molasses at second level (40g/L).

Table 5- Amount of reduced weight of soil samples in v=18km/h

Sample number	Weight of sample before wind tunnel testing	Weight of sample after wind tunnel testing	Reduction percent
1	23.5	22.85	2.77
2	15	14.7	2
3	35	34	1.45
4	31	30.7	0.97
5	27	26.2	2.96
6	25	24.7	3.61
7	18	16.5	12.5
8	16.3	15.7	3.68
9	20.3	18.7	7.88
10 (first control)	29.2	28.4	2.74
11(second control)	33	30.3	8.18

Table 6- Effect of different level of factors on soil stabilization by *Bacillus* sp.

Factors	Level 1	Level 2	Level 3
NH_4Cl	2.523	2.513	4.353
Urea	1.49	3.136	4.313
Molasses	3.096	3.873	1.969

Figure 3a shows effect of ammonium chloride (nitrogen source) on soil fixation. There was a low amount of soil stabilization (2.073) in level 1, and this amount raised (2.513) in level 2, and there was a maximum amount by 4.353 in level 3.

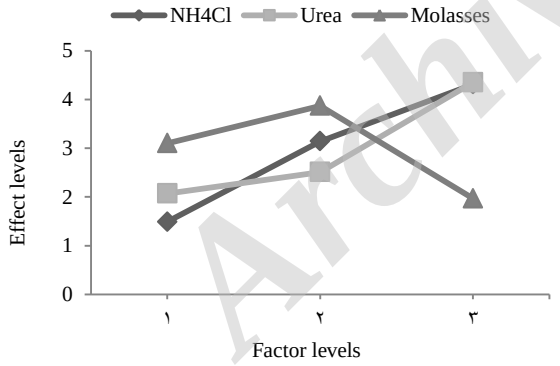

Fig. 3- Effect of different levels of NH_4Cl, urea and molasses on soil stabilization by *Bacillus* sp.

Effect of urea factor on soil stabilization is illustrated in figure 3b. Amount of soil stabilization increased form level 1by 1.49 to level 3 by 4.313. This result indicates that urea has direct effect on soil fixation. Also, effect of molasses on soil stabilization is demonstrated in figure 3c. In this case, there was increasing of soil stabilization form level 1 (3.096) to level 2 (3.873), but in level 3 (1.969), reduction of this process was observed.

Interaction of factors on soil stabilization by *Bacillus* sp. HAI4: Table 7 shows factors interaction on soil stabilization. Interactions of paired factors were evaluated based on amount of selected two factors which was changeable form 0.86 to 24.67. Maximum interaction was 24.67 for NH_4Cl×molasses that NH_4Cl was in level 3 and molasses was in level 2. Also, Interaction of urea×molasses had minimum intensity of interaction (0.86) as urea in level 3 and molasses in level 2. NH4Cl and urea in level 3 had 8.82 of interaction.

Variance analysis (ANOVA) for soil stabilization by *Bacillus* sp. HAI4: Variance analysis (ANOVA) illustrates effective parameters on soil stabilization by *Bacillus* sp HAI4. Table 8 demonstrates molasses factor had not effect but NH4Cl and urea factors had higher effect by 1.869 and 11.412 respectively. Maximum effect is related to urea which shows a major role of nitrogen in soil fixation.

Estimation of optimum conditions for soil stabilization: Table 9 illustrates share of each factor in soil stabilization. Based on these results, urea and NH_4Cl factors have higher and lower shares. Expected result in optimum conditions of soil stabilization was -0.428%. NH_4Cl and urea in level 1 and molasses in level 3 had suitable levels.

Table 7- Interaction effect of paired factors on soil stabilization by *Bacillus* sp.

Number	Effect of paired factors	Interaction of factors	Intensity of interaction factors	Column	Optimum conditions
NH_4Cl×molasses	1	1×3	24.67	2	2.2
NH_4Cl×urea	2	1×2	8.82	3	2.1
Urea×Molasses	3	2×3	0.86	1	1.2

Table 8- Variance analysis in soil stabilization by *Bacillus* sp.

Factors	DOF (f)	Sum of squares (S)	Variance (V)	F-Ratio (F)	Pure Sum (S')	Percent (%)
NH_4Cl	2	8.777	4.388	1.076	0.644	1.869
Urea	2	12.067	6.033	1.483	3.934	11.412
Molasses	2	5.495	2.747	0.675	0	0
Total	8	34.473				100%

Table 9- Optimum conditions for soil stabilization by *Bacillus* sp. HAI4

Column/Factor	Level description	Level	Contribution
NH_4Cl	Factor A-Level 1	1	-0.907
Urea	Factor B-Level 1	1	-1.49
Molasses	Factor C-Level3	3	-1.011
Total contribution from all factors			-3.409
Current grand average of performance			2.98
Expected result at optimum condition			-0.428

Discussion and conclusion

Today, stabilization of soil and dust by microorganisms is important and because of climate change, desertification and dust storm can effect on environment and human health (28). Stabilization of soils by microorganisms can be effected by different factors such as calcium, pH, and temperature and urea concentration. Therefore, improvement of soil stabilization by microorganisms can lead to increasing microorganisms capacity in soil stabilization (1).

Results of this study illustrated that Taguchi method is one of the effective methods for optimization of effective factors in soil stabilization process. In this method, three factors (NH_4Cl, urea and molasses) were surveyed in soil fixation optimization. In this case, urea concentration had higher effect on soil stabilization and calcite sedimentation by *Bacillus* sp HAI4. Also, interaction of two factors shows dependence of factors together.

Meyer and coworkers (2011) used NH_4Cl with 10g/L concentration as nitrogen source for *Sarcina pasteurii* in soil stabilization (29). This group observed that soil samples as a treatment in wind tunnel by 40 km/h wind velocity had higher mass than controls. In this investigation, three levels (0.5, 1 and 1.5 g/L) were determined for this factor in which the highest effect (4.353) in soil stabilization was related to level 3 of NH_4Cl (Table 6). Therefore, by increasing NH_4Cl concentration, soil stabilization increased (Fig. 3). The reason of this change is high ammonium anion for rising of pH, urea hydrolysis and calcite sedimentation.

An important process in ecosystems ammonium oxidation is carried by autotrophic ammonia-oxidizing bacteria (AOB) and change in diversity and quantity of total bacteria is resulted from the application of urea to the soil (30). Also, Neg and coworkers (2012) applied 0.25M of urea for *Bacillus megaterium* in order to stabilize soil samples. In our study, three levels 20, 30, and 40 g/L of urea were utilized. Results illustrated that effective

level was 3 with 4.313% (14). Therefore, it can result in a way that by increasing urea concentration, soil stabilization increases. Hereon, urea hydrolysis was augmented and leads to calcite sedimentation. Similarly, Dejong et al (2006) illustrated suitable result of soil stabilization in sandy samples by using 20 g/L of urea for *Sarcina pasteurii* (31). Amount of 250 mmol in groundwater samples for *Sarcina pasteurii* was used in order to close fracture and porosity in stones for inhibition of groundwater flows and contaminant transport.

Molasses is another factor that was evaluated in soil fixation. Molasses with 1 g/L was applied for *Sarcina pasteurii* in order to prevent water leakage in groundwater. Taguchi design in this study, as three levels of molasses (30, 40 and 50 g/L) illustrated maximum soil stabilization in level 2 by 3.873%. In fact, by increasing of molasses, soil stabilization firstly increased and then decreased (Figure 3). Before initiation of urea hydrolysis, molasses resulted in biomass augmentation; therefore this process causes pH decrease. Therefore, molasses increasing does not have an effective role in soil fixation.

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ارزیابی خاک پایدارشده بوسیله *Bacillus* sp. HAI4 در شرایط مختلف از طریق روش تاگوچی

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چکیده

مقدمه: فرایند رسوب کلسیت، در نتیجه فعالیت‌های متابولیکی میکروارگانیسم‌ها، کلسیفیکاسیون زیستی نامیده می‌شود. در این فرایند، کلسیت ($CaCO_3$) به وسیله فعالیت‌های میکروارگانیسم‌ها بر روی دانه‌های خاک ته‌نشین شده و سبب افزایش سختی/ استحکام و کاهش فرسایش خاک می‌شود.

مواد و روش‌ها: در این مطالعه، میزان هیدرولیز اوره و ته‌نشینی کلسیت در فرایند تثبیت خاک، بوسیله باکتری *Bacillus* sp. HAI4 از طریق روش تاگوچی مورد ارزیابی قرار گرفته است. همچنین، تونل باد برای اندازه‌گیری میزان پایداری خاک، طراحی شد.

نتایج: در این پژوهش، سه فاکتور (کلرید آمونیوم، اوره و ملاس) برای بررسی تثبیت خاک، بررسی شدند. نتایج این مطالعه، نشان داد که روش تاگوچی یکی از کارامدترین روش‌ها برای بررسی فاکتورهای موثر در فرایند پایداری خاک است. در این مورد، غلظت اوره، دارای بیشترین تاثیر در رسوب‌دهی کلسیت و تثبیت خاک بوسیله این باکتری بود. همچنین، بررسی اندرکنش فاکتورها، ارتباط فاکتورها را با هم نشان داد. در مورد فاکتور کلرید آمونیوم با سه سطح (۰/۵، ۱ و ۱/۵ گرم بر لیتر)، در سطح سوم، بیشترین تاثیر(۴/۳۵۳٪) را در فرایند تثبیت خاک داشت. افزایش کلریدآمونیوم، باعث افزایش میزان پایداری خاک شد. سه سطح ۲۰، ۳۰ و ۴۰ گرم بر لیتر اوره نیز مورد بررسی قرار گرفت. دراین مورد نیز، سطح سوم با میزان۴/۳۱۳٪ بیشترین تاثیر را داشت. همچنین، در این مطالعه، طراحی تاگوچی، به صورت سه سطح ملاس (۳۰، ۴۰ و ۵۰ گرم بر لیتر)، بیشینه تثبیت خاک را در سطح دوم با میزان ۳/۸۷۳٪ نشان داد. در حقیقت، ابتدا با افزایش ملاس، تثبیت خاک، افزایش و سپس کاهش یافت.

بحث و نتیجه‌گیری: در حالت کلی، این مطالعه نشان داد که دو فاکتور کلرید آمونیوم و اوره، تاثیر بیشتری در میزان هیدرو لیز اوره و رسوب کلسیت دارند.

واژه‌های کلیدی: *کلسیفیکاسیون زیستی، پایداری خاک، .Bacillus sp ، رسوب کلسیت، آمونیوم کلراید*

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