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Improvement of sand consolidation, compressive strength of mortar and absorption reduction of water using $CaCo_3$ – precipitating bacteria

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The ureolytic bacterial isolate ALEX1 able to precipitate $CaCO_3$ was isolated from a local urea-rich soil. Due to it's a relatively high urease activity, isolate ALEX1 was tested for its ability to improve consolidation of sand, compressive strength of mortar and absorption reduction of water. The data revealed that the highest values of compressive strength and reduction of water absorption were 16.53% and 88.59%, respectively. Identification techniques showed that isolate ALEX1 is a Bacillus cereus strain. Its urease pure enzyme showed a maximum activity at a reaction pH of 7.0, and a relatively high activity in a pH range of 7.0 to 8.0. The enzyme was stable at heat treatment up to 60ºC. The amino acids composition of the purified enzyme showed that aspartic was the major amino acid followed by leucin, arginine and glutamic. The results indicate possibility of using Bacillus cereus cells in bioremediation and self-healing of soils.

Keywords: *Bacillus cereus*, biocementation, urease, comprehensive strength, water absorption, consolidation

INTRODUCTION

Due to its numerous applications, $CaCO_3$ precipitation has attracted much research attention. These applications include fixation of the atmospheric carbon dioxide, dolomite precipitation, capture of the inorganic pollutants, concrete crack remediation, conservation of stone artworks and extracellular biocementation using carbonate-producing microorganisms (Rodriguez-Navarro et al., 2003). Through their metabolic pathways, several organisms can precipitate $CaCO_3$. These organisms include; photosynthetic cyanobacteria and algae, sulphate-reducing bacteria and organisms involved in the nitrogen cycle (Castanier et al., 1999). In the metabolic processes, photosynthetic organisms utilize dissolved CO_2 to form $CaCO_3$ (Ehrlich, 1998). Sulphate-reducing bacteria can reduce sulphate to H_2S and HCO_3 in the presence of organic matter and absence of oxygen (Wright, 2002). However, in the nitrogen cycle the involving organisms, ammonification of amino acids, reduction of nitrate and hydrolysis of urea are the most investigated $CaCO_3$ precipitation processes (Fujita, 2000).

The most studied of all above described mechanisms is the hydrolysis of urea by the enzyme urease (Warren et al., 2001). Ureases are a set of enzymes common in nature between plants, bacteria, fungi, algae and invertebrates that although with diverse protein structures, exercise a single catalytic purpose, that is the hydrolysis of urea (H_2N-CO-NH_2), its final yields being ammonia and carbonic acid. The activity

and quantity of urease for $CaCO_3$ precipitation are based on several environmental factors including bacterial cell concentrations, bacterial type, pH, temperature, urea and calcium concentrations (Mortensen et al., 2011; Qabany et al., 2012). Many bacterial genera including *Pseudomonas*, *Bacillus*, *Proteus*, and *Myxococcus* known as urease producers (Rodriguez-Navarro et al., 2003). However, high urease production activities and the ability to reduce urea in the presence of ammonia with high stability are the required properties of an ideal microbial source used for the purposes of biocementation.

In the present study, we report the screening, isolation and identification of an indigenous strain of *Bacillus cereus* with capability of $CaCO_3$ precipitation that may be suitable as an environmentally friendly technique in the concrete industry. This was achieved by testing bacterial isolates for their ability to precipitate $CaCO_3$, measuring urease activity of isolates, testing their ability to improve the consolidation of sand and compressive strength of mortar as well as absorption reduction properties.

MATERIALS AND METHODS

Screening for $CaCO_3$-precpitatating bacteria

To isolate bacteria with high level of urease activity, environmental samples were collected from different Egyptian ecosystems including water (fresh and marine) and agriculture soil (field fertilized and unfertilized with urea) from February to June 2015 (Table 1). The collection of samples was done using sterile instruments under aseptic conditions.

Screening was performed as previously described by (Al-Thawadi et al., 2011) with slight modification of the media composition. One gram of soil or 1 ml of water was placed in 50 ml of growth media in 250 ml shaking flasks, at 30°C, for 24 h. The enrichment medium was containing 30 mM $CaCl_2$ in nutrient broth consisting of (g/l); 3, urea; 20, $NaHCO_3$; 2.12, NH_4Cl; 10. To screen for pure colonies, the enrichment cultures were diluted and plated out on nutrient agar plates, pH 7. After autoclaving, 8% of filter-sterilized urea was added to the plates and incubated at 30°C for 48 h. Then, the filter sterilized urease activity indicator phenol red was placed on the surface of the colonies and the change in the color was observed. After urea hydrolysis, due to the increase in the pH up to 9.0, the pinkish color appeared surrounding the ureolytic bacteria. The individual colonies that showed a pinkish color and formed crystals seen by naked eye were purified by streaking on a solid medium of the same composition and then isolated on urea-agar slants. Six bacterial colonies were selected for further studies based on the crystal formation and urease activity test.

Selection of the highly ureolytic bacteria

Selection of the highest ureolytic bacteria among the six isolates was performed. Determination of urease was carried out according to Weatherburn (1967), the reactions were performed in microtubes containing 100 µl of sample, 500 µl of 50 mM urea and 500 µl of 100 mM potassium phosphate buffer (pH 8.0) giving a total volume of 1.1 ml. The reaction mixture was incubated at 37°C for 30 min in a shaking water bath. The reaction was stopped by transferring 50 µl of reaction mixture to the tubes containing 500 µl of phenol–sodium nitroprusside solution (0.05 g sodium nitroprusside, 1 g phenol/100 ml distilled water).

Table (1): Samples and sampling sites

Sample Name	Sample Type	Governorate
Agricultural field fertilized with urea	Fertilized Soil	Elmonifya
Agricultural field	Soil	Kafr Elda-war
Agricultural field	Soil	Kafr- Elshikh
Agricultural field fertilized with urea	Fertilized soil	Elmonifya
Mariout Lake	Lack water	Alexandria
Abu -Qir Bay	Lack water	
EL-Shatby beach	Sea water	
Al Malahaate	Salt Mine Soil	
Garden soil	Soil	

Five hundred µl of alkaline hypochlorite (3.56 g Na_2HPO_4, 1 ml sodium hypochlorite in 100 ml distilled water) was added to the tubes and incubated at room temperature for 30 min. Finally, the optical density of the color complex was measured in a spectrophotometer at 630 nm against the control (500 ml phenol nitroprusside sodium, 500 ml sodium hypochlorite, 50 ml distilled water) and compared to a standard curve prepared generated with $(NH4)_2SO_4$. Controls used for the enzyme reactions were a reaction mixture without substrate and a reaction mixture without incubation. One unit of urease activity was defined as the amount of enzyme liberating 1 mg NH_3 from urea per minute, under the above assay conditions. Out of the urease assay results, isolate ALEX1 was selected for biocementation assays and the other isolates were excluded.

Biocementation experiments

Sand consolidation assay

Before the assay, the four tested isolates were cultivated in nutrient broth at 35°C for 24 h in a shaker incubator with 170 rpm. According to that described by Stabnikov et al., (2011), two components of liquid biocement were applied sequentially as follows: i) cultural liquid (bacterial suspension) with addition of $CaCl_2$ to a concentration of 0.1 M; ii) calcium chloride and urea solution contained 82.5 g/L (0.75 M) of $CaCl_2$ and 60 g/L (1M) of urea. Solutions of urea and calcium were mixed with water and injected to initiate the biocementation process. The biomass was determined by measurement of optical density (OD) spectrophotometrically at 660 nm. Core for cementation consisted of 100 ml plastic cup that was contained in the bottom with glass filter paper and the cup was drilled from the back to allow the cementation solution to pass through filter paper to another plastic cup which is considered as a collector of solution. Eighty grams of sand was dried packed in a drilled plastic cup under continuous vibration to yield an even density. Core was up-flushed with water and tapped to remove air pockets. Ca/urea (cementation) solution and cells were premixed immediately before injection into the core by pouring both liquids into cup containing sand. The solution was poured on sand daily until the process was finished after 7 days from the start of the operation.

Compressive strength test

To study the effect of urease producing strains on compressive strength test of cement mortar, ALX1 and ALX2 isolates which showed strong positive sand consolidations were grown in NB media. Isolates were suspended in saline buffer and phosphate solution to a final concentration of 1.2 $x10^8$/ml. Mortar cubes with cementation solution and tap water containing no cells were prepared and regarded as control 1 and control 2, respectively. Five hundred grams of Al-Arish Portland cement (CEM/B 52.2), 1500g local sand and 250ml solution (cementation solution and buffer containing bacteria isolates) were thoroughly mixed by using standard mortar mixer 65-L0005. The mixture was cast in Hydraulic shrinkage of cement mortar 65-L0010/B of dimensions (4x4x16cm) and was left for 24 h. After that all specimens were cured in tap water at room temperature until compression testing with different intervals. The intervals of all specimens with saline buffer were 7, 14 and 28 days, and the intervals of specimens with phosphate buffer were 3, 21 and 28 days. After each interval days the cubes were removed from the curing water and cut to four mortars with dimension to 7x7x7 cm^3 by using ELECTRIC TILE SAW. Control specimen was also prepared in a similar way where bacterial culture substituted water. The cubes were then placed in the compression testing machine without any packing and a load of pace rate 2.9 was applied. The load was increased until the cubes cracked and the strength recorded in kilo Newton (kN) along with the average stress recorded in N/mm.

Water absorption test

This test was performed according to Sarda et al., (2009). All mortar specimens were cured in tap water for 28 days, saturated overnight in water and weighed. The bricks were then dried in an oven at 100°C for 24 h, cooled and weighed again. Water absorption was calculated by using following formula**:**

$$\%\text{Water absorption} = W_{saturation} - W_{oven\ dried} / W_{oven\ dried}$$

Where $W_{saturation}$ is the weight of bricks after saturation in water for 24 h, and W_{Oven} dried is the weight of bricks after oven drying for 24 h. Out of all above assays, isolate ALEX1 was the highly biocement producer and ureolytic bacteria and selected for further characterization and identification.

Molecular, biochemical and physiological characterization of isolate ALEX1

For molecular identification, genomic DNA of isolate, ALX1 was extracted using AMSHAG DNA extraction kit (Redwan and Abd-El-Haleem, 2005), which was then used as the template for 16S rDNA amplification. Primers used in the 16S rDNA amplification were 27F and 1492R **(**Redwan and Abd-El-Haleem, 2005**)**. The amplification process was conducted in a PCR machine using Maxima Hot Start PCR master mix (Thermo K1051). The used PCR cycles were as follow: 95°C for 10 min; 35 cycles of 95°C for 30 s, 65°C for 1 min, and 72°C for 1.5 min; and final extension at 72°C for 10 min. The purified PCR amplicons were sequenced by ABI 3730 DNA sequencer (Applied Biosystems). Thereafter, the obtained sequences were aligned with corresponding sequences from related organisms, which were retrieved from the GenBank database using the BLAST algorithm. The sequence of strain ALX1 was submitted to the GenBank and had an accession number.

Isolate ALX1 was also subjected for identification using different characteristics according to Bergye's Manual of Determinative Bacteriology (Hensyl, 1994). Tests of indole, methyl red reaction, Voges-Proskauer (V.P.) reaction, citrate utilization, oxidase, catalase, casein hydrolysis, starch hydrolysis, and motility test were performed as reported by (Chester and Cooper, 1979). For physiological experiments, the optimum pH, growth temperature and NaCl tolerance were determined.

Purification of urease enzyme and determination of its amino acids composition

Culturing of strain ALEX1 was performed as described above. Partial purification of the crude urease present in culture filtrate of strain ALEX1 was carried out by fractional precipitation with 50% ammonium sulphate as described by (Wingfield, 2016). Further purification of the ammonium sulphate fraction was achieved by gel filtration chromatography on Sephadex G-200 (Pristoupil, 1995). To determine the purity and molecular weight, SDS-page was performed as described by (Laemmli and Fever, 1973). The peak which gave the highest urease activity was considered as a pure enzyme. The amino acid composition of the purified urease enzyme was hydrolyzed in vacuum in 6 N HCl at 105°C for 24 h and analyzed by using 4700 MS/MS mode: positive MS-MS mode (Macrogene, Korea).

RESULTS

Screening and selection of CaCO3-precipitating bacteria

Based on crystals formation, protein content and urease activity assay, six positive bacterial isolates ALEX1-ALEX6 were selected, purified and preserved. The results presented in Table 2 indicated that all isolates were able to degrade urea and produce urease enzyme with diverse levels. However, isolates ALEX1 was the most effective urea degrader with urease activity 2.21 U/mg and protein content 1.25 mg/ml, respectively. On the other hand, the lower urease activity was obtained with isolate ALEX6. No positive isolates were obtained from water samples. On urea agar plates, only isolates ALEX1, ALEX2, ALEX3 and ALEX4 showed a rapid and intense pink color development indicating its urea hydrolysis ability by urease enzyme. Isolate ALEX1 was the highest and selected for biocementation experiments.

Biocementation experiments

Sand consolidation

To confirm the pre-screening results for $CaCO_3$ precipitation, the selected isolate ALEX1 were examined for their ability to consolidate sand. Photograph, light microscopy and SEM analysis on microbial involvement in sand consolidation is depicted in Figure 1A-C. As shown in Figure 1A, in comparison with the control, a high crust formation through sand consolidation on the top layer of the sand positive consolidation was noticed. The presence of built calcite crystals among sand particles is shown obviously in Figure 1B. From SEM observations (Figure 1C), $CaCO_3$ (indicated by red arrows) precipitated by bacterial cells could be clearly distinguished, while it's not observed in the control made without any addition of bacterial cells.

Compressive strength

As shown in Figure 2, compressive strength of saline buffer media was significantly increased with all mortar cubes that contained bacterial cells of strains ALEX1 at all tested curing times. However, in phosphate buffer, the compressive strength was down at curing time 7 and 14 days, while an increase was observed at 28 days. The compressive strength of isolates ALEX1 improved by 14.87% in saline buffer and 16.53% in phosphate buffer comparing to the controls.

Water absorption

The water absorbance test was conducted to

determine the increase in resistance towards water penetration in mortars cubes in the presence and absence of bacterial cells. As shown in Figure 3, by treating the mortar cubes with bacteria and a calcium source, decreased water absorption was observed compared to the untreated specimens (controls).

Table 2: Samples, location, urease activity, protein content and crystal formation of positive $CaCO_3$-precipitating isolates

Samples	Sampling site	Urease Activity U/ml	Protein content (mg/ml)	Crystals formation after 6 days of incubation	Urea hydrolysis
Alex1	Urea fertilized soil samples collected from Elmonifya governorate, Egypt	2.21	1.25	High	+
Alex2		1.48	1.14	medium	+
Alex3		0.98	0.98	medium	+
Alex4	Unfertilized soil samples collected from a house garden, Alexandria, Egypt	0.81	0.92	weak	+
Alex5		0.73	0.86	weak	-
Alex6		0.64	0.75	weak	-

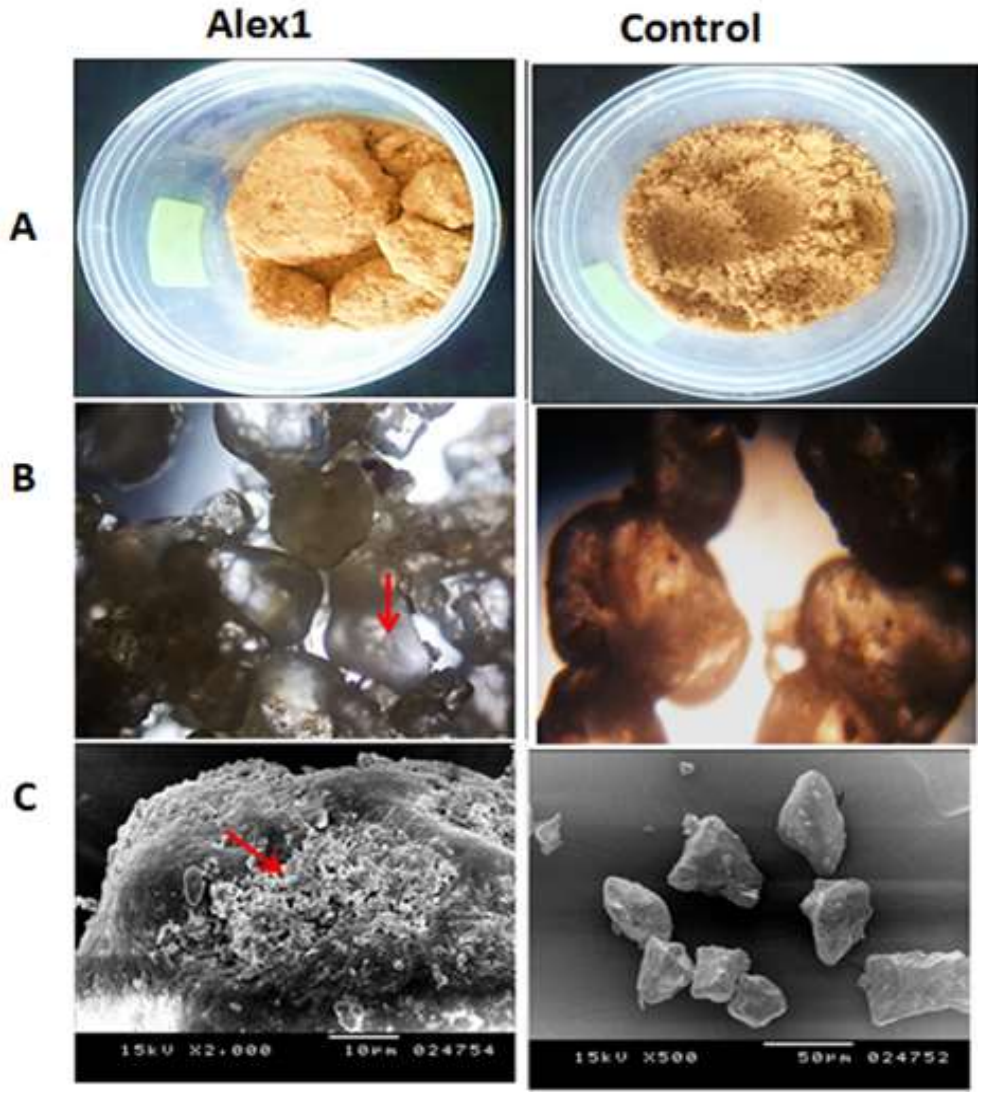

Figure 1: Crust formation (A), light microscopy (B) and SEM analysis (C) of sand consolidation assay occurred using isolates ALEX1 and control (without bacterial culture), respectively.

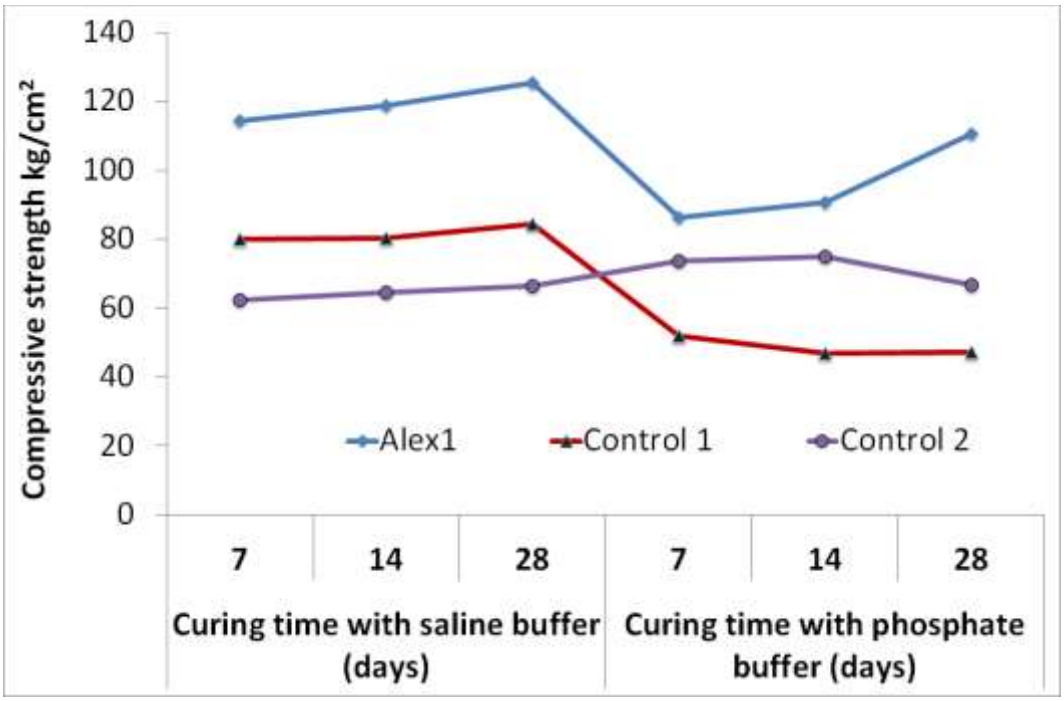

Figure 2: Compressive strength of cement mortar with and without bacterial cells in saline and phosphate buffers of strains ALEX1 and ALEX2 up to 28 days.

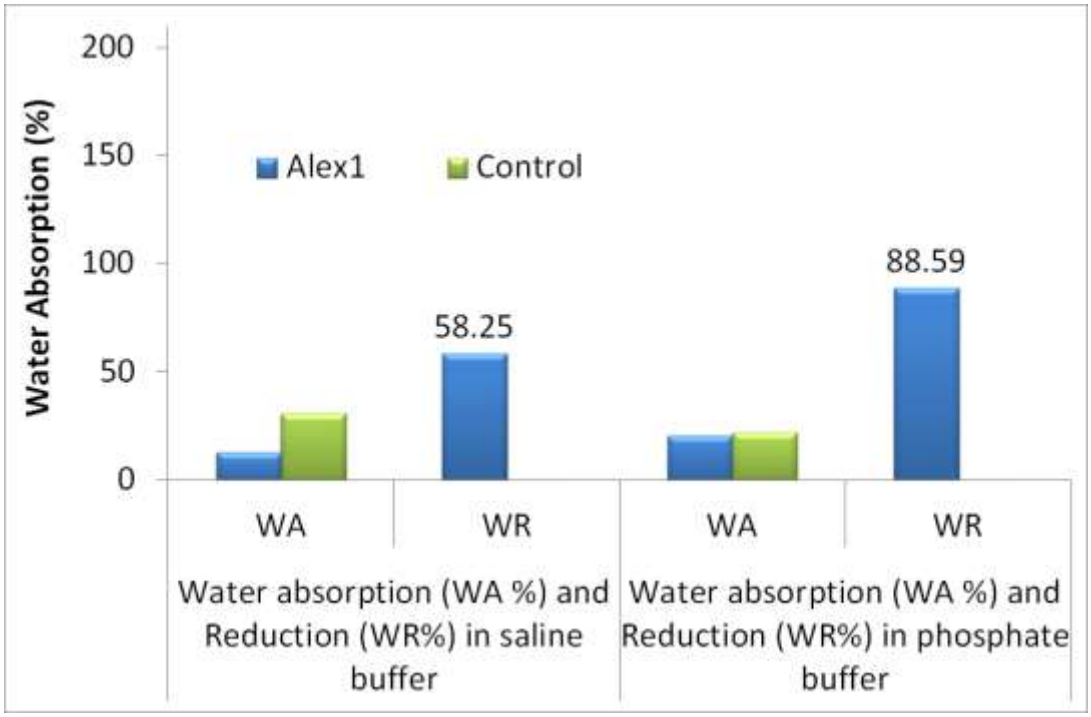

Figure 3: Ratio (%) of water absorption and reduction in the presence/absence of bacteria (ALEX1 and ALEX2) with saline and phosphate buffers.

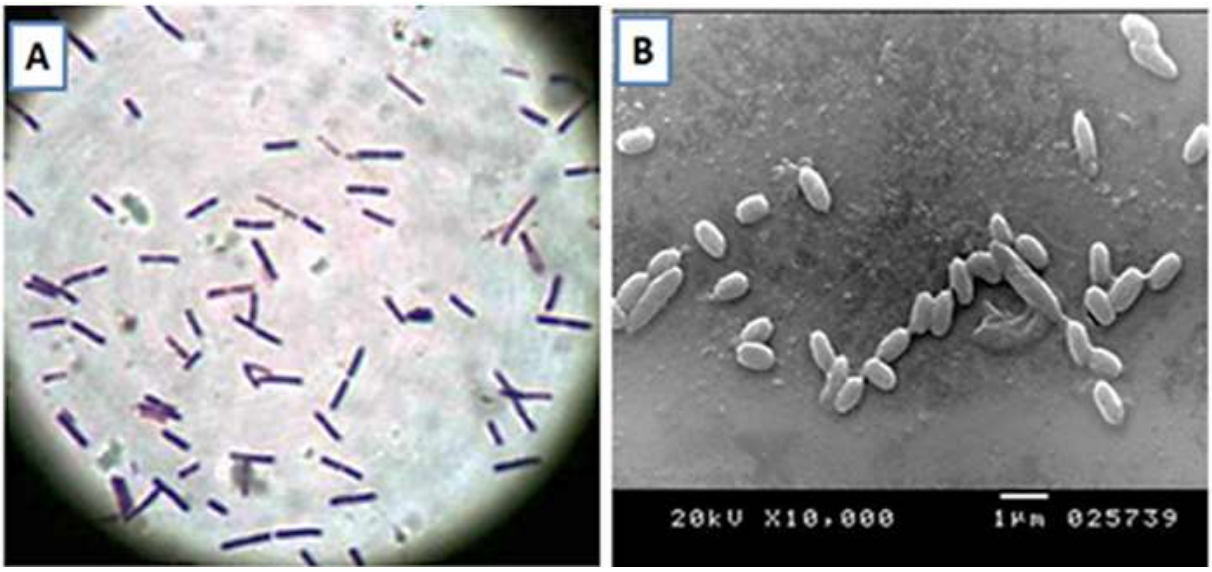

Figure 4: Cells of isolate ALEX1 under oil immersion (A) and SEM (B), respectively.

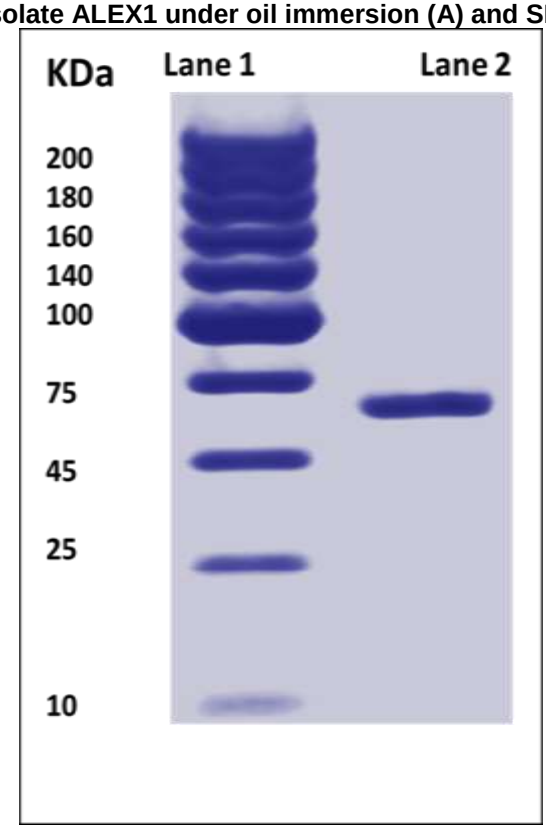

Figure 5:SDS-PAGE of purified urease fraction from strain *Bacillus cereus*. Lane 1: molecular protein weight markers (KDa). Lane 2: purified enzyme fraction.

Table 3: Morphological, physiological and biochemical characterization of isolate Alex

Character	Alex 1
Colony characteristics	
Shape	Round
Color	Creamy
Margin	Entire
Elevation	Slightly
Consistency	Slightly sticky
Opacity	Opaque
Microscopic features	
Gram stain	+ve
Cell shape	Rods
Sporulation	Spore former
IMViC test	
Indol	-ve
Methyl red test	-ve
VP test	+ve
Citrate utilization test	-ve
Nitrate rduction	+ve
Sugar utilization	
D-Maltose	+veVe
Dextrose	+ve
D-Cellobiose	-ve
Trehalose	-ve
Sucrose	-ve
D-Raffinose	-ve
α-D-Lactose.0	-ve
D-Mellibiose	-ve
Arabinose	+ve
Galactose	+ve
Xylose	+ve
Ribose	+ve
Sorbitol	-ve
Adonitol	-ve
Inulin	-ve
Mannose	-ve
Rhamnose	-ve
Glycrol	-ve
Fructose	-ve
Gas production in all	-ve
Enzyme production test.	
Amylase	-ve
Protease	+ve
Lipase	-ve
Cellulase	-ve
Galactase	+ve
Gelatinase	+ve
Urease	+ve
Oxidase	+ve
Catalase	+ve
Pectinase	-ve
Caseinase	+ve
Physiological characterization	
Temperature growth	30-50^{o}C
Optimum temperature	37^{o}C
pH growth range	7-12
Optimum pH	7
NaCl tolerance range	0-4 %
Optimum NaCl conc.	1 %
H2S production	**-ve**

Table 4: Amino acid composition of the purified urease enzyme

Amino acids	Relative concentration (%)
Glycine	1.84
Lysine	9.39
Valine	8.65
Aspartic	18.41
Leucine	15.10
Arginine	13.99
Glutamic acid	16.57
Proline	10.16
Theronine	4.97
Methionine	0.92

Moreover, the water absorption capacities of mortars cube using both saline and phosphate buffers were decreased compared to the controls. The maximum reduction in water absorption test using saline buffer was 58.25%, while it was 88.59% with phosphate buffer for isolate ALEX1, respectively.

Morphological, biochemical and Molecular identification of isolate ALEX1

According to the results of assays described above, strain ALEX1 was selected for identification and its urease enzyme for intensive characterization. As presented in Table 3, isolates ALEX1 was shown to be Gram-positive rod with spore formation. It gave positive results with catalase, oxidase, urease, VP test and nitrate reduction, but negative results were noticed with MR test, indole tests and H_2S production. Growth was observed at NaCl concentrations ranging from 0% to 4%. The isolate was able to assimilate most of the sugars as carbon source. It grows in pH range 7-12, the isolate was found to grow optimally at temperature range of 30-50^{o}C. Based on these data isolate ALEX 1 is recommended to be *Bacillus cereus*. To confirm these results, isolate ALEX1 were subjected for light and scanning electron microscopy (SEM). Figure 4 showed the morphology of the isolate.

To confirm these identification results, strain ALEX1 was subjected for molecular identification and its 16S ribosomal RNA nucleotide sequence was determined. Using the alignment search tools (http://blast.ncbi.nlm.nih.gov), the 16S DNA of strain ALEX1 showed homology of >99% with the corresponding DNA of the bacterial species *Bacillus cereus*. So, strain ALEX1was submitted to the GenBank under the Accession number of KY750252.

Characterization of urease enzyme produced by strain ALEX1

As shown in Figure 5, SDS-PAGE analysis of the Ureolytic active fractions collected during gel filtration showed mainly 1 protein band in the molecular mass of about 60 KDa. The amino acids composition of the purified fraction presented in Table 4 indicated that aspartic acid has the highest relative concentration (18.41%) followed by leucin, arginine and glutamic acids. On the other hand, methionine, glycine and threonine showed the lowest percentage of amino acids with relative concentrations 0.92%, 1.84 and 4.79%, respectively.

DISCUSSION

The main task of the microbial induced carbonate precipitation (MICP) is isolation and selection of active urease producing bacteria that can prompt $CaCO_3$ precipitation in sand consolidation and biocementation processes (Kang et al., 2014). Biocementation is to develop the strength and rigidity properties of soil and rocks though microbial activity (Knoll, 2003). Traditional grouting methods for ground development employ particulate (cement/bentonite) or chemical mortars that can be rather expensive and environmentally unfriendly. Recently, novel grouting techniques have been advanced to treat unsaturated coarse-soils by stimulating natural processes (Neupane et al., 2013). One of these methods, termed biogrouting, has offered some opportunities in soil cementation via (MICP). This way simulates natural process by depositing calcite ($CaCO_3$) on the soil grains, thereby increasing the material's stiffness/strength.

In the present study, we screened for bacterial isolates able to produce urease and investigate their ability to perform as a catalyst in a

biocementation process. Among six isolates, isolate ALEX1 displayed the highest growth and protein content; therefore, it was selected as the test organism. Pre-screening for $CaCO_3$ precipitating bacteria by urease enzyme was performed using UAB medium. Usually, this medium used for separation of a variety of microorganisms, especially on the basis of urease production and used in several studies for isolation, differentiation and determination of ureolytic microorganisms able to precipitate $CaCO_3$ (Achal and Pan, 2011; Chahal et al., 2011).

Isolate ALEX1 showed positive results to consolidate and shear strength the majority of calcite precipitation in upper most surface area of the sand column. This might be due to the higher growth of bacteria in the presence of oxygen which subsequently induces active precipitation of $CaCO_3$ around the surface area. Similar results were reported in sand by Al-Thawadi (2011). The effect of bacterial calcite precipitation on the compressive strength of cement mortar cubes after 7, 14 and 28 days was assessed. The greatest improvement in compressive strength occured in phosphate buffer, there was 16.53% compared to the control cement mortar. However, the reduction in compressive strength of the cubes having saline buffer may be due to the existence of chloride ions in the solution, which is known to decline the integrity of the cement matrix reported previously by (Berke et al., 1988).

It is a known fact that many other factors affect the development of strength of concrete and consequently its durability other than curing or the curing technique applied. These factors include quality and quantity of cement used in a mix, grading of aggregates, maximum nominal size, shape and surface texture of aggregate (Arum and Alhassan, 2005) water/cement ratios, degree of compaction and the presence or otherwise of clay particles and organic matter in the mix (Arum and Udoh, 2005). The high compressive strength recorded at the 28^{th} day can be attributed to curing acting a vital role on strength increase and stability of concrete. Curing takes place immediately after concrete placing and finishing, and includes preservation of desired moisture and temperature conditions, as curing period was increased, microbial cells started growing slowly. Upon cell growth, calcite would have precipitated on the cell surface as well as within the cement mortar matrix. Once the pores in the matrix were plugged, the movement of the nutrients and oxygen to the bacterial cells were stopped. Finally, the cells either died or turned into endospores, which led to increasing the compressive strength of the mortar cubes. This explains the increased compressive strength at the age of 28 days in cement mortar cubes prepared with ALEX1 microbial cell (Raheem and Abimbola, 2006).

In concern to the water absorption assay, mortar cubes treated with bacteria and a calcium source exhibited a significant decrease of the water uptake compared to the control. Maximum reduction in water absorption test was 88.59% with phosphate buffer. Similar observations were reported by De-Muynck et al. (2008).

Out of the above results and intensive screening strain ALEX1 was selected and identified as *Bacillus cereus* strain ALEX1. *Bacillus* group is a common type of bacteria used for the production of urease and calcite precipitation. For example, *Sporosarcina pasteurii* was the central organism used for multiple applications such as remediation of heavy metals and radionuclides, crack remediation in concrete and soil improvement (Whiffin et. al., 2007; Sarada et al., 2009; Gorospe et al., 2013; Lauchnor et. al., 2013; Li et al., 2013), while *B. megaterum* was used to improve the concrete strength and stability of building materials and structures (Siddique et al., 2008; Soon et al., 2013; Dhami et al., 2014). Therefore, it is interesting to add another Bacillus species to perform a biocementation process.

Amino acid composition of the purified urease enzyme produced by strain ALEX1 was studied and the result indicated the presence of 10 amino acids. Aspartic was the major amino acid of the enzymes with a relatively high amount of leucin, arginine and glutamic. These results are in agreement with (Hu and Mobley, 1990) who reported that the analysis of urease from *Helicobacter pylori* had significantly higher asparagine plus aspartic acid content than the other enzymes. On the other hand, methionine and glycine showed the lowest percentage of amino acids in the purified urease enzyme of *Bacillus cereus* ALEX1. Absence of either member in the purified urease enzyme might be possible. This is probably because its content in the enzyme is too small to be detected (Gulati et al., 1997).

CONCLUSION

In conclusion, present study provides an ureolytic bacterium so called *Bacillus cereus* strain ALEX1 that can prompt $CaCO_3$ precipitation

and could be employed in multiple applications such as bioremediation, consolidation and cementation.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

HM, GA, AY and ME designed and performed the experiments and also wrote the manuscript. DA, SE and SZ designed experiments, performed data analysis and reviewed the manuscript. All authors read and approved the final version.

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