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Leveraging the concretes calcification with carbonic anhydrase produced by *Alcaligenes faecalis* GA(B) (Mn847724.1)

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A R T I C L E I N F O

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A B S T R A C T

Concrete is an integrated substance that is composed of cement, water, and aggregates. The aim of this study was to biocalcify concretes with bacterial carbonic anhydrase (CA). To achieve this, a soil sample taken from a construction site in Minna Nigeria, was serially diluted and plated on nutrient agar with $CaCO_3$ added. After 7, 14 and 28 days of curing by water immersion, the compressive strength of the concrete was determined and the effects of CA on the concretes were investigated. *Alcaligenes faecalis* was identified as the isolate with the better ability for CA production. The CA has a mean activity of 0.0351 U/ml, an increase in substrate concentration on CA activity from 1 to 7 mM, an optimum temperature of 50 °C and pH of 8.5. Concretes calcified with CA from *A. faecalis* gained a strength of 48.29 % after 28 days of curing by water immersion. The results demonstrated that CA has an enormous potential in concrete biocalcification.

1. Introduction

Concrete is an essential material that is employed for building as well as construction of infrastructure owing to the fact that, they are relatively cheap, easily accessible and allow casting into varying shapes and sizes. Concrete is often described as materials made up of cement, fine and coarse aggregates, and water (Chen et al., 2023; Tang et al., 2023). Concrete is however, sensitive when there is imbalance in the settings, thus, causing changes such as formation of cracks. These cracks in turn reduce the durability of concrete, as such, often requires repairs leading to more cost. The cracks formation occurs due to lack of fortification as a result of poor tensile strength (Luhar et al., 2022). As a result, during casting, steel is added in other to accommodate and support the expanded weight (Tittelboom et al., 2010).

Whenever concrete encounters high tensile stress, it results in deformation among other effects, caused by corrosion of the steel utilized as reinforcement. Cracks created in concrete makes it vulnerable and exposes it to exogenous substances from the environment including various gases and liquids, which most times seeps through the cracks and reacts with the concrete's composition. Thus, the concrete as well as reinforcement materials over time undergoes various degrees of damages, which ultimately results in structural failure or collapse. In simple terms, cracks in concrete are the major cause of failure in the architecture of civil engineering structures and failure to act on time or initiate appropriate repairs will further broaden the cracks, which will eventually require additional cost for the repairs as well as maintenance (Neha et al., 2018).

Conventionally, mechanisms used for repairs of compromised concretes majorly depend on the process where materials of different kinds and characteristics are matched together, including the composition of inorganic and organic calcium hydrosilicate as well as petroleum epoxies. Alternatively, a viable approach utilizing bacteria and or their metabolites can be utilized in the repairs as well as strengthening of concrete (Rosewitz et al., 2021). This method has been developed and

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employed in the past for strengthening of concrete as well as healing of cracks through a technique known as biocalcification or microbiologically induced carbonate precipitation (MICCP). This technique involves a process that selectively performs fillings that are mediated by microorganisms where calcium carbonate ($CaCO_3$) precipitation serves as a sealant through the actions/metabolic activities of the microorganisms (Rohini and Padmapriya, 2023). This process ultimately results in the reduction of gaps and other openings as well as improve the durability and compressive strength of the concrete (Gandhimathi et al., 2015; Mahat et al., 2023).

Microorganisms are ubiquitous and a large reservoir of biochemical substances, which are very essential in production of enzymes. Enzymes of microbial origin are robust naturally; they are more stable to heat and pH. They also possess varying functions, thus, making them an ideal candidate that can be employed in various biotechnological processes (Thapa et al., 2019). There has been a gradual development and public acceptance in the use of enzymes in biotechnology, which has been greatly attributed to their functional features such as high rate of catalysis, efficiency, and selectivity. Thus, making them a useful candidate in a broad range of industrial processes, which are not harmful to the environment (Robinson, 2015). Enzymes from microorganisms are of high caliber and are mostly preferred to enzymes from other sources (i.e., plants or animals); and are easily produced into large quantities. Furthermore, the physicochemical and physiological properties of the microorganisms can be manipulated as well as regulated to obtained desired results with little cost in the extracellular extraction and purification (Vachher et al., 2021).

Carbonic anhydrases (EC 4.2.1.1) are metalloenzymes containing zinc; this enzyme can catalyze the conversion of CO_2 to bicarbonate and also have the capacity to catalyze the hydration reversible reaction of CO_2 ($CO_2 + H_2O \Leftrightarrow HCO_3^- + H^+$) (Angeli et al., 2020). The active sites of CAs are composed of Zn^{2+}, which is arranged and held together by three histidine residues, a hydroxide ion complex with one molecule of H_2O. The process involved in the interconversion of CO_2 and bicarbonate ions is facilitated by the catalytic action of CAs, causing the transfer of ions of hydrogen through the active sites of CA as well as the surrounding buffer, which leads to pH alteration when the reaction progresses to a state of equilibrium, raising the pH from 8.2 to 8.4. Calcium, $CaCO_3$ or calcite ions is eventually formed with CA providing the enabling environment composed of available and appropriate pH and cation responsible for the formation of calcite (Komala and Khun, 2014). CAs can function as an esterase as well as hydratase in the formation of carbonate. The whole processes involved in the hydration of CO_2 has been identified to be the rate determinant in the precipitation of $CaCO_3$ reaction enabled by the availability of Ca^{2+} (Muller et al., 2013).

The process of calcite precipitation is sped up by the actions of CA under conditions that are favorable (Zhang et al., 2011). The actions of CA and the resultant by-products function as sealant using calcite produced to close up gaps and cracks present in concretes. The mechanism behind the gaps closure is a function of the precipitated calcite; the calcite initiates a self-assemblage on the surfaces of concrete as well as inside the cracks and crevices through the production of stable crystalline substances known as the calcite precipitate. In the presence of calcium, the CA can be employed to rapidly form $CaCO_3$. The properties of $CaCO_3$ produced is similar to that of conventional cement used in construction of concrete. The CO_2 present in the atmosphere get sequestered during the catalytic reaction of CA resulting in $CaCO_3$ precipitation. The whole sequestration process is environmentally friendly with little or no adverse effects to man or the ecosystem at large. Most CA have been reported in the past to be dependent on/contain Zn^{2+}.CA is also dependent on Fe^{2+} and Cd^{2+} (Rosewitz et al., 2021).

Concrete with cracks can be improved upon (i.e., increasing the compressive strength, stiffness and filling of cracks) using biological technique. The effect of biocalcification can be felt when calcite is produced by bacterial cells either endogenously or exogenously. More so, changes in the chemistry of the solution are attributed to the metabolic activities involving mineral precipitation (Patil et al., 2016). There are various types of bacteria that can cause calcite precipitation, among them are *Bacillus megaterium* and *Bacillus sphaericus*, which have been regarded generally as safe since they are not pathogenic. These bacteria also thrive at pH of 9.0, which is optimum for its metabolic activities, although, the pH is harsh for the survival of other bacteria. These *Bacillus* species form spores, which afford them the capacity to stay longer in cements or concrete in the form of capsules where they excrete exopolysaccharide substances that enhances the adhesion of components of concrete (Whitaker et al., 2018; Agereh et al., 2019).

Reports have been made regarding the potentials of CA produced by calcifying bacteria; the biological catalyst they produced are often equipped in catalyzing the precipitation of $CaCO_3$ from hydration of CO_2 whenever calcium is present in the environment (Bansal et al., 2016). MICCP is a good biotechnological technique that is regarded as safe to the environment. It is efficient in causing calcite precipitation without depleting natural resources; the final product does not emit harmful/hazardous substances. As such, its usage in construction company has been highly recommendable since it facilitates a stronger and a denser concrete (Satinder et al., 2017).

Carbon dioxide (CO_2) and other gas concentrations are rising in the atmosphere as a anthropogenic activities, particularly industrialization (Supuran, 2022). A number of significant events, including global warming, climate changes, changes in rainfall, and an increase in sea level, were as a result of increase in CO_2. The CO_2 is released into the atmosphere via different routes, but the largest source is the burning of fossil fuels for transportation, industry, generating electricity, and construction of structures. The main industrial operation that contributes to atmospheric CO_2 is the manufacture of cement. Land use change, in particular, deforestation is responsible for12% anthropogenic CO_2 emissions, cement manufacturing is responsible for 88 % of them (Alshalif et al., 2019).

Through a process known as bio-mineralization, biological sequestration of CO_2 can naturally transform CO_2 into biominerals e.g. calcium carbonate and magnesium carbonate (Alshalif et al., 2019; Abatenh et al., 2018). A bacterial enzyme called carbonic anhydrase (CA) was used in this instance to capture and convert CO_2 to $CaCO_3$. The ability of CA to catalyze the hydration of CO_2 to HCO_3^-, which can precipitate in the form of carbonate as $CaCO_3$ and has improved safety, stability, and eco-friendliness, allowing for the safe release of CO_2 without harming the environment (Dhami et al., 2013; Chang et al., 2017). Further to this, CO_2 enters the concretes and is absorbed thereby interacting with the cement in the presence of moisture to produce $CaCO_3$ (Alshalif et al., 2019). Therefore, this research was aimed at producing carbonic anhydrase from bacterial isolates found in soil for the biocalcification of concrete.

2. Materials and methods

2.1. Samples collection

Samples of soil utilized for this research were obtained from two selected concrete construction sites in Minna-Nigeria, sited between *Latitude: 9° 36′ 54.86″ N and Longitude: 6° 32′ 51.94″*. The soil samples were taken to the Microbiology Laboratory of Federal University of Technology, Minna, Nigeria for microbiological analysis.

2.2. Preparation of the para-nitrophenyl acetate (pNPA)

A 25 mg of para-nitrophenyl acetate (pNPA) salt was dissolved in 2 ml of acetone, using the protocol employed by Ibrahim et al. (2016). A 3 mM of the salt concentration was prepared through the addition of sterilized distilled water, and aseptically stored in a sterile bottle.

2.3. Isolation and screening of bacteria

A 10-fold serial dilution of the soil samples was carried out following the slightly modified protocols of Li et al. (2004). Inoculation of 1 ml aliquot from dilution 3 (10^{-3}) was done using a sterile syringe. Sterile molten nutrient agar (18 ml) was poured aseptically into the Petri plate containing the inoculated soil sample. The plate was gently swirled clockwise and anticlockwise to ensure the contents are distributed evenly. Upon solidification, 3 mM of p-NPA was used in spraying the surface of the culture plate before incubating for 48 h. Two distinct colonies of bacteria that developed (isolates GAA and GAB) were carefully picked before streaking on sterile NA plate so as to obtain pure isolates.

2.4. Confirmatory test for CA production

The bacterial isolates obtained were inoculated into test tubes containing 5 ml of nutrient broth and 3 mM of p-NPA following the method of Alshalif et al. (2019). A control was set up (without bacterial isolate), after which the inoculated test tubes were incubated in a shaker for 14 days at 37 °C.

2.5. Characterization and identification of bacterial isolates

The method of Cheesbrough (2006) was utilized for characterizing the bacterial isolate while the protocols of Frank et al. (2008) were used to determine the molecular identity of the isolate. The gene sequence of the isolate with a better potential for CA production (isolate GAB) was submitted to the NCBI GenBank and the provided accession number was MN847724.1. The following sections describe the detailed protocol for the molecular identification of the isolate:

2.6. DNA extraction

Pure cultures of the bacterial sample were transferred to 1.5 ml of liquid medium and cultures were grown on a shaker at 28 °C for 48 h. This was followed by the centrifugation of the cultures for 5 min at 4600*g*, and the re-suspension of the resultant pellets in 520 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Fifteen microliters (15 μl) of 20 % SDS and 3 μl of Proteinase K (20 mg/ml) were then added, and the mixture was incubated at 37 °C for 1 h, followed by the addition and vortexing of 100 μl of 5 M NaCl and 80 μl of a 10 % CTAB solution in 0.7 M NaCl. The suspension was then incubated at 65 °C for 10 min and placed on ice for 15 min. This was followed by the addition of an equal volume of chloroform: isoamyl alcohol (24:1) which was then incubated on ice for 5 min and centrifuged for 20 min at 7200*g*. The aqueous phase was then moved to a new tube, adding isopropanol in a ratio of 1:0.6 and the DNA precipitated for 16 h at −20 °C. Collection of the DNA was carried out by centrifugation for 10 min at 13000*g*, washed with 500 μl of 70 % ethanol, air-dried for 3 h at room temperature (28 °C ± 2) and finally dissolved in 50 μl of TE buffer (Frank et al., 2008).

2.7. Polymerase chain reaction (PCR)

The cocktail of preparation for PCR sequencing comprised 10 μl of 5× GoTaq colourless reaction, 3 μl of 25 mM $MgCl_2$, 1 μl of 10 mM of dNTPs mixture, 1 μl of 10 pmol each 27 F 5′-AGA GTT TGA TCM TGG CTC AG-3′ and -1525R, 5′-AAGGAGGTGATCCAGCC-3′ primers and 0.3 units of Taq DNA polymerase (Promega, USA) made up to 42 μl with sterile distilled water and 8 μl DNA template. PCR was performed in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) with a PCR profile comprising of an initial denaturation for 5 min at 94 °C, followed by 30 cycles made up of 94 °C for 30 s, 50 °C for 60 s and 72 °C for 90 s; and a final termination for 10 min at 72 °C, and kept at 4 °C GEL (Frank et al., 2008).

2.8. Integrity test

To validate the amplification, the integrity of the amplified DNA of about 1.5 Mb gene fragment was tested on an Agarose gel of about 1 %. The buffer (1XTAE buffer) was prepared and subsequently used to prepare 1.5 % agarose gel. The suspension was boiled for 5 min in a microwave. The molten agarose was then allowed to cool to 60 °C and stained with 3 μl of 0.5 g/ml ethidium bromide (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was placed into the casting tray slots and the molten agarose was transferred into the tray. The gel was allowed to stand for 20 min in order to solidify to form the wells. The 1 × TAE buffer was poured into the gel tank to barely cover the gel. Two microliter (2 μl) of 10× blue gel loading dye (which gives colour and density to the samples to make it easy to load into the wells and keep tab on the progress of the gel) was added to 4 μl of each of the PCR product and loaded into the wells after the 100 bp DNA ladder was loaded into well 1. The gel was electrophoresed for 45 min at 120 V, visualized by ultraviolet trans-illumination and photographed. The sizes of the PCR products were determined by comparison with the mobility of a 100 bp molecular weight ladder that was ran alongside experimental samples in the gel (Frank et al., 2008).

2.9. Purification of amplified product

The amplified fragments were ethanol purified in order to remove the PCR reagents. Briefly, 7.6 μl of 3 M Sodium acetate and 240 μl of 95 % ethanol were added to 40 μl PCR amplified product in a new sterile 1.5 μl Eppendorf tube, it was mixed thoroughly by vortexing and kept at −20 °C for 30 min. Centrifugation for 10 min at 13000*g* and 4 °C was followed by removal of the supernatant (by inverting the tube on trash once) after which the pellets were washed by adding 150 μl of 70 % ethanol and mixed, then centrifuged for 15 min at 7500*g* and 4 °C. Again all supernatant was removed (by inverting tube on trash) and then also by inverting the tube on paper tissue and allowed to dry in the fume hood at room temperature (28 °C ± 2) for 10–15 min. It was then resuspended with 20 μl of sterile distilled water and kept in −20 °C prior to sequencing. The purified fragment was checked on a 1.5 % Agarose gel and ran on 110 V for about 1 h, to confirm the presence of the purified product and quantified using a nanodrop (model 2000, Thermoscientific, USA) (Frank et al., 2008).

2.10. DNA sequencing analysis

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer (Applied Biosystems, USA) using manufacturers' manual while the sequencing kit used was that of BigDye terminator v3.1 cycle sequencing kit. Bio- Edit software and MEGA 6 were used for all the genetic analysis.

2.11. Inoculum preparation for large scale preparation of crude CA

The method of Li et al. (2004) was used to prepare a 100 ml basal medium in a 250 ml conical flask following manufacturer's instructions. The medium was autoclaved for 15 min at 121 °C, and then allowed to cool. The pure bacterial isolates were inoculated into the broth using a sterilized wire loop. The conical flask was incubated for 96 h at 30 °C in a rotary shaker running at 150 rpm.

2.12. Basic culture medium

The method of Zhang et al. (2011) was used to formulate the culture medium used for the production of carbonic anhydrase (CA). It contained (g/L) sucrose 5.0, disodium hydrogen phosphate 2.0, magnesium sulphate 0.5, calcium carbonate 0.1, Iron (III) chloride 0.005 and cement 5, in 1 L of distilled water. The medium was autoclaved for 15 min at 121 °C. After cooling, 20 % (v/v) inoculums from the previously

prepared inoculum were added. The culture was incubated for 7 days at 32 °C and 150 rpm in a rotary shaker. The culture medium was spun for 15 min at 7000 rpm to obtain a supernatant. The supernatant was then collected and filtered through a 0.22 μm pore size filter membrane to obtain a cell free supernatant. The cell free supernatant is the crude CA produced by *Alcaligenes faecalis* G (Mn847724.1).

2.13. Determination of CA activity and optimum parameters for CA activity

The procedure described by Muller et al. (2013) was used to determine the enzyme activity. A reaction mixture consisting of phosphate buffer and 3 mM pNPA was prepared, and 0.1 ml of crude CA was added to 0.9 ml of the prepared reaction mixture in a test tube. Blank preparation served as the control. The solutions were incubated at 37 °C for 5 min, measurements were made for 5 min with a UV spectrophotometer using a 412 nm wavelength and colour change was observed. Para-nitrophenol (pNP) was appropriately dissolved in test tubes at concentrations of 1–5 mg/ml of dH_2O to produce the pNP curve.

Para-nitrophenylacetate (pNPA) was employed to find the appropriate substrate concentration for the best CA activity. The enzyme was incubated at various substrate concentrations (1 mM to 7 mM) for 5 min, and then the absorbance was determined at 412 nm. Para-nitrophenylacetate (pNPA) was also employed to find the ideal temperature for CA activity. The enzyme was incubated for 5 min at a temperature between 30 and 80 °C, and then the absorbance at 412 nm was determined. It was done again for pH values of 5.5, 7.5, 8.5, and 9.5. Three runs of each reaction were carried out for each test. The generated results were utilized to draw a standard curve, and the pNP standard curve was used to compute the CA activity. The enzyme activity was expressed in U/ml (Capasso et al., 2012; Panchami et al., 2019).

2.14. Concrete mixing and casting of concrete cubes

An iron mould with an internal dimension of 150 mm^3 was used in moulding concrete in accordance with the BS 1881 (1993). The mix design for the concrete was carried out manually using the Nominal Concrete mix ratio of 1:2:4 for cement: sand: aggregate mix and then 80 % CA solution (with activity of 0.0351 U/ml) in 20 % water was added. A concrete mixture with no CA (100 % water) was prepared to serve as the control. The concretes were made into cubes, with the dimension of 150 mm × 150 mm × 150 mm and labeled. Further information on the mix design for the concrete is presented in Table S1.

Further information on the mix design for the concrete is presented in Table S1. Before filling the mould with adequately prepared concrete, it was lubricated with oil. The concrete was mixed in three layers each and tampered 25 times. The concrete was properly finished and labeled for proper identification. The cubes were then left for 24 h to set before demoulding as shown in Fig. 1a. After demoulding the concrete cubes, they were all placed into a curing tank containing clean water and allowed for curing for specific period of time (7, 14 and 28 days) as presented in Fig. 1b.

2.15. Compressive strength testing

The concrete cubes were removed from the curing tank, air-dried, weighed and placed axially in the crushing machine such that at least two faces of the cube are in direct contact with the crushing machine's plate as instructed in part 116:1983 of B.S., 1881 (1993). The compressive strength of the concrete was determined in conformation with IS 516:1959 and calculated as follows:

$$\text{Compressive strength} = \frac{\text{Load of failure}}{\text{cross sectional area of the cube.}}$$

Gain in percentage (%) strength was calculated as:

$$\frac{\text{Compressive strenth at day 28} - \text{compressive strenght at day 7} \times 100}{\text{Compressive strenght at day 28}}$$

2.16. Scanning electron microscopy (SEM)

Scanning electron microscopy (EVO LS 10, CARL ZEISS, Germany) was utilized in viewing the surface morphology and the calcite precipitate composition on the concrete specimen. Concrete samples for SEM were collected from the broken pieces of cubes obtained from compressive strength test. The concrete samples were first dried using Hexamethyldisilazane (HMDS) and at a temperature of 50 °C in an oven to remove any trace of moisture for about 6–8 h before infiltration and subsequent embedding of the samples. The concrete specimens were mounted on a stub of metal with the aid of a sticky carbon disc to increase the conductivity rate. In order to prevent the buildup of charges on the surface of the concrete specimens, it was coated with gold (using a Denton Vacuum Desk IV coating system) of about 10 nm in thickness, which was applied controllably in a sputter coater before the examination.

The concrete samples were then loaded into the SEM with the help of aluminum subs, and then placed in the stage or sample holder. The samples were imaged under a high vacuum at a current of 10.00kv, 100 μm lens aperture and at 11.0 mm working distance. Collection of the images was done with the aid of the secondary electron detector with an acquisition time/image of 2 min and 40 s, and the images were 2560 × 1920 pixels each. Lastly, the recorded SEM images ranged from 3000× to 20,000×, and the results recorded for interpretation

2.17. Data analysis

One-way analysis of variance (ANOVA) was utilized in the analysis of data obtained from this study using SPSS version 23. Statistical significance was considered at P values < 0.05 while statistical insignificance was considered at P values > 0.05.

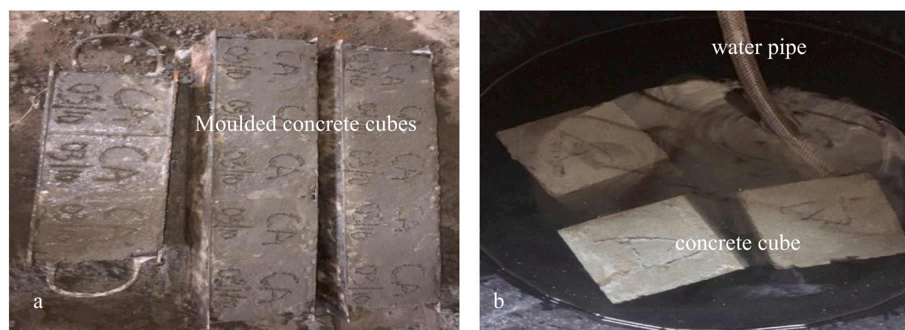

Fig. 1. (a) Moulded concrete cubes (b) Demoulded concrete cubes cured by water immersion.

3. Results

3.1. Potentials of producing carbonic anhydrase by bacterial isolates

Two bacterial isolates screened for their CA producing abilities were presented in Fig. 2a and b. The yellow colonies observed in Fig. 2a indicated the hydration of para-nitrophenylacetate (pNPA), thus confirming the ability of the isolate to produce CA, while in Fig. 2b, there was a complete hydration to give orange/peach colonies.

3.2. Characterized and identified CA producing bacteria

The result of the morphological and biochemical characterization is presented in Table 1.

3.3. Molecular identity of isolate GAB

The molecular identity of the isolate GAB confirmed it is 99.9 % similar to *Alcaligenes faecalis* subsp. *parafaecalis* Strain G. The image of the agarose gel electrophoresis is presented in Fig. 3a. The phylogenetic relationship of isolate GAB with other related organisms is shown in Fig. 3b. This shows a similarity of the isolate with other organisms including *Salmonella enteric*, *Escherichia coli*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

3.4. CA activity and optimum substrate concentration for CA activity

The mean enzyme activity for carbonic anhydrase by isolates GAA and GAB were 0.0321 and 0.0351 U/ml respectively, thus showing that isolate GAB had a higher enzyme activity compared to isolate GAA. Thus, isolate GAB was selected for the production of the enzyme and for calcification of the concrete. The enzyme activity for substrate optimization is shown in Table 2.

The results showed a gradual increase in the enzyme activities as the substrate concentration increases from 1 mM to 7 mM. The optimum temperature for CA production by the isolates was at 50 °C while the least was at 80 °C (Table 3). pH 8.5 was optimum for the enzyme production whereas pH 5.5 was the least of the pH range examined (Table 4). The result of the crushing strength gained by the bioconcretes after 28 days is shown in Table 5. Of the two treatments, concretes calcified with CA gained a strength of 48.29 % compared to control with strength gained of 9.19 % after 28 days of curing by water immersion.

Table 1
Cultural, microscopic and biochemical characteristics of CA producing bacterial isolates.

Observations	Bacterial isolates	
	GAB	GAA
Form	Circular	Circular
Size	Medium	Small
Colour	Orange-peach	Yellow
Texture	Mucoid	Mucoid
Margin	Entire	Entire
Elevation	Flat	Raised
Microscopy	Bacilli	Bacilli
Biochemical tests		
Gram's reaction	−Rod	+Rod
Vogues Proskauer	+	+
Citrate	−	−
Hydrogen sulphide production	−	−
Catalase	+	−
Spore	−	+
Anaerobic incubation	−	+
Coagulase	−	−
Oxidase	+	−
Indole	−	−
Motility	+	+
Carbohydrate utilization:		
Fructose	Gp, Ap	Gp, Ap
Maltose	− +	− +
Glucose	+ +	− +
Arabinose	− −	− +
Lactose	− −	− +
	− +	− +

Key: − (Negative), + (Positive), Gp (Gas production), Ap (Acid production).

3.5. Crushing strength

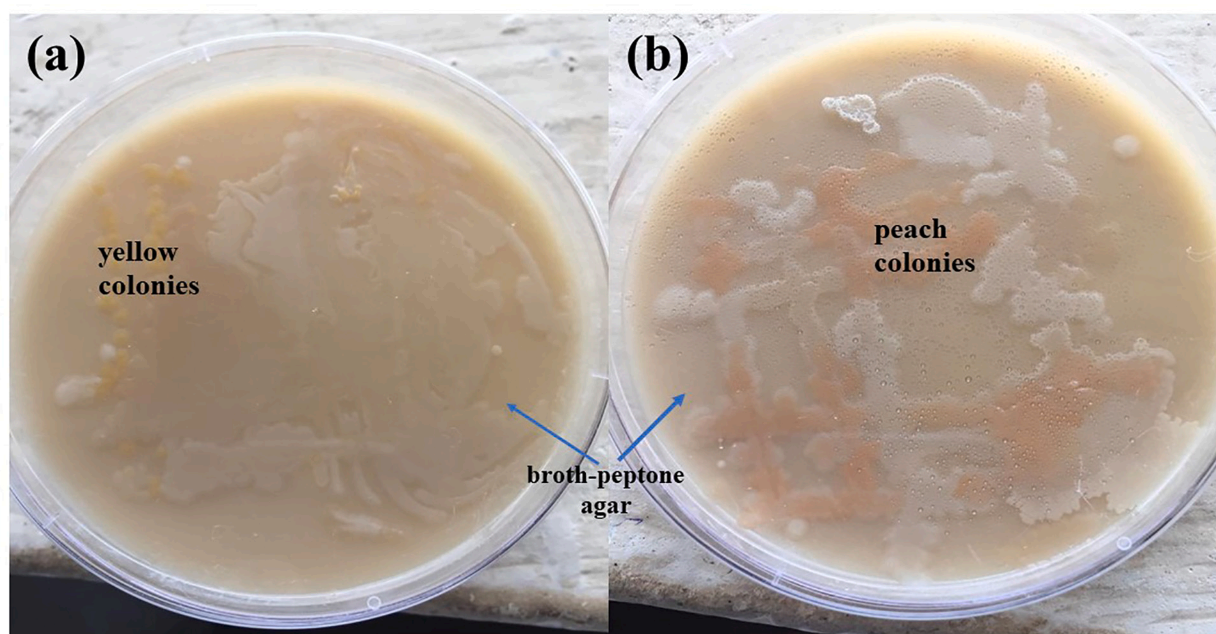

Fig. 2. Reaction of isolates GAA (a) and GAB (b) to pNPA.

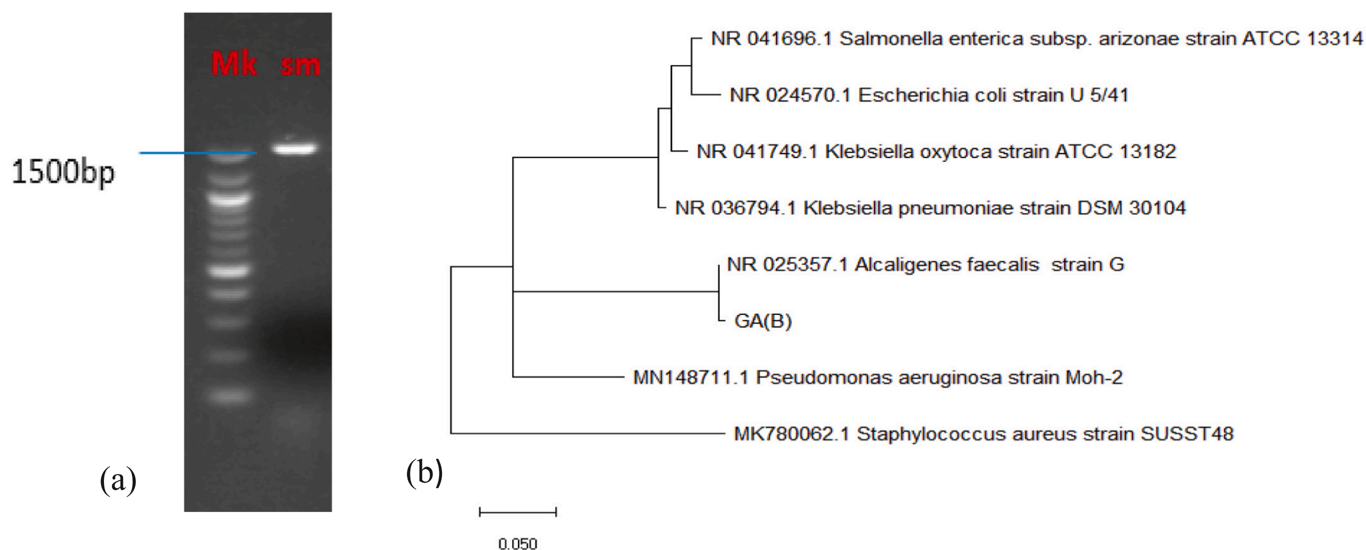

Fig. 3. Gel image of isolate (a) and phylogenetic relationship of isolate GAB with other selected bacteria (b). (Key: Mk = DNA Ladder 1500 bp, Organism's approximate size = 1500 bp, sm = Sample (*Alcaligenes faecalis* subsp. *parafaecalis* Strain G)).

Table 2
Effect of substrate concentration on CA activity.

Substrate concentration (mM)	GA(A)	GA(B)
1	$0.0115^{a} \pm 2.3 \times 10^{-4}$	$0.0133^{a} \pm 1.4 \times 10^{-4}$
2	$0.0224^{b} \pm 1.7 \times 10^{-4}$	$0.0246^{b} \pm 8.8 \times 10^{-4}$
3	$0.0321^{c} \pm 1.4 \times 10^{-4}$	$0.0351^{c} \pm 2.4 \times 10^{-4}$
4	$0.0433^{d} \pm 1.4 \times 10^{-4}$	$0.0510^{d} \pm 8.7 \times 10^{-4}$
5	$0.0440^{e} \pm 1.4 \times 10^{-4}$	$0.0675^{e} \pm 6.5 \times 10^{-4}$
6	$0.0433^{d} \pm 1.4 \times 10^{-4}$	$0.0703^{f} \pm 2.6 \times 10^{-4}$
7	$0.0436^{de} \pm 1.5 \times 10^{-4}$	$0.0709^{f} \pm 1.7 \times 10^{-4}$

Mean values with same letter in the same column do not differ significantly at $P > 0.05$.

Table 3
Effect of temperature on CA activity.

Temperature (°C)	GA(A)	GA(B)
30	$0.0246^{b} \pm 8.82 \times 10^{-5}$	$0.0290^{b} \pm 8.82 \times 10^{-5}$
40	$0.0304^{c} \pm 8.82 \times 10^{-5}$	$0.0316^{c} \pm 1.15 \times 10^{-4}$
50	$0.0329^{d} \pm 1.15 \times 10^{-4}$	$0.0370^{e} \pm 1.45 \times 10^{-4}$
60	$0.0306^{c} \pm 1.45 \times 10^{-4}$	$0.0350^{d} \pm 6.08 \times 10^{-4}$
70	$0.0306^{c} \pm 1.2 \times 10^{-4}$	$0.0314^{c} \pm 3.48 \times 10^{-4}$
80	$0.0194^{a} \pm 8.82 \times 10^{-5}$	$0.0244^{a} \pm 3.33 \times 10^{-5}$

Table 4
Effect of pH on CA activity.

pH	GA(A)	GA(B)
5.5	$0.0106^{a} \pm 8.82 \times 10^{-5}$	$0.0119^{a} \pm 8.82 \times 10^{-5}$
6.5	$0.0325^{b} \pm 8.82 \times 10^{-5}$	$0.0337^{b} \pm 1.12 \times 10^{-3}$
7.5	$0.0355^{c} \pm 8.82 \times 10^{-5}$	$0.0355^{bc} \pm 1.10 \times 10^{-3}$
8.5	$0.0357^{c} \pm 8.82 \times 10^{-5}$	$0.0371^{cd} \pm 1.41 \times 10^{-3}$
9.5	$0.0352^{c} \pm 8.82 \times 10^{-5}$	$0.0385^{d} \pm 1.04 \times 10^{-3}$
10.5	$0.0320^{b} \pm 8.82 \times 10^{-5}$	$0.0337^{b} \pm 1.2 \times 10^{-4}$

3.6. Scanning electron micrographs of the precipitated $CaCO_3$

The results of the concretes as viewed using the scanning electron microscope at 100 μm is presented in Fig. S1. The CA calcified concrete (b) had crystals of $CaCO_3$ compared to the control- without CA (A) which had no indication of $CaCO_3$ deposits.

Table 5
Mean concrete cube compressive strength for Grade 25 concrete.

	Crushing strength (N/mm²)		
	Expected strengths (standard)	Control	CA (bioconcrete)
7	16.25	$20.46^{a} \pm 11.43$	$11.54^{a} \pm 4.09$
14	22.50	$23.11^{a} \pm 0.25$	$15.52^{ab} \pm 0.52$
28	24.75	$22.53^{a} \pm 1.2$	$22.28^{b} \pm 1.70$
Percentage strength gained	–	9.19 %	48.29 %

4. Discussion

The two bacteria isolated from soil of construction sites in this study (GAA and GAB) had the potentials of producing carbonic anhydrase (CA), due to the fact that they were able to produce pNP from the hydrolysis of pNPA. Isolate GAA was able to produce distinct colonies with yellow coloration on NA agar plate. Furthermore, isolate GAB was observed to produce an orange/peach coloration. The yellow colonies (from isolate GAA) were as a result of the isolate's ability to yield acetate and pNP from the hydrolysis of pNPA, which is similar with the result obtained from the study of Li et al. (2004) whose bacterial isolates were obtained from the Karst region in China. The orange/peach colonies on the contrary could be a product of excessive pNPA hydrolysis by the CA produced by the bacteria.

Similarly, a change in colour of the nutrient agar to a dense yellow colour from the initial light yellow is a product of the pNPA hydrolysis by the bacterial isolates. This is in line with the result reported by Alshalif et al. (2019) where the ability to produce CA was determined by the potential of the Gram-negative bacteria to hydrolyze pNPA. Results from isolate GAB molecular characterization revealed a 99 % similarity with *Alcaligenes faecalis* subsp. *parafaecalis* strain G. The enzymatic activity of crude CA was higher (0.0351 ± 0.0002 U/ml) in CA produced by *A. faecalis* subsp. *parafaecalis* strain G compared to 0.03210 ± 0.012 U/ml produced by isolate GAA.

The optimum substrate concentration for crude CA production was reported to be 5 mM (0.0440 U/ml) and 7 mM (0.0709 U/ml) for isolate GAA and *A. faecalis* subsp. *parafaecalis* strain G respectively. The reason

for the increased enzymatic activity with increased substrate concentration for the CA could be due to the ability of the isolate to withstand and degrade higher concentration of pollutants, including heavy metals (Basharat et al., 2018).

The optimum temperature recorded in the production of crude CA was reported at 50 °C for both isolates with an enzyme activity of 0.0329 U/ml and 0.0370 U/ml for isolate GAA and *Alcaligenes faecalis* subsp. *parafaecalis* strain G respectively. This result obtained was in accordance with that of Muley et al. (2014), where the temperature of 50 °C was reported to be optimum for CA activity (0.0362 U/ml) produced by *Bacillus schlegelii* while working on atmospheric CO_2 sequestration. The crude CA produced by *Alcaligenes faecalis* subsp. *parafaecalis* strain G was found to have an optimum pH of 9.5 with the mean enzyme activity recorded as 0.0385 U/ml whereas a pH of 8.5 was reported for isolate GAA with the mean CA activity as 0.0357 U/ml. Again, these results were similar to the one reported in the study of Muley et al. (2014), where the pH of 10.0 was reported for the optimum CA activity produced by *B. schlegelii* while working on atmospheric sequestration of CO_2. However, these findings were contrary to the study reported by Demir et al. (2001), where the optimum pH of CA ranges between 6.5 and 7.5 for CA activity from erythrocyte of human, which was similar to erythrocyte from plasma membrane of bovine. The variation in pH between the microbial CA and that from human and bovine cells were attributed to genotypic variations in the organisms.

Increase in curing time of concrete showed a relative increase in the crushing strength. This may be due to the length of time given for continuous hydration of the concretes to proceed. This leads to hardening of concretes, thereby improving the compressive strength. It was observed that on day 7, the crushing strength was 11.54 N/mm^2, which increased to 15.52 and 22.28 N/mm^2 on the 14th and 28th day respectively. A similar increase in the crushing strength relative to increase in curing days was also observed in the study of Lagazo et al. (2019) which utilizes *B. subtilis* enhancing the strength of concrete. In this study, the crushing strength of concrete increased from 14.89 N/mm^2 reported on day 7 to 16.42 and 19.26 N/mm^2 on day 14 and 28 respectively (Lagazo et al., 2019). By comparing between percentage strength of both concrete, the control (concrete without CA) and the test concrete (concrete treated with CA), it was observed that over a 28-day period, the control concrete gained a percentage strength of 9.19 while the test concrete (bioconcrete) had a percentage strength of 48.29 %.

No precipitate of $CaCO_3$ was visible at a resolution of 100 μm when the SEM micrograph of the control specimen (A) was observed. However, SEM micrograph of bioconcrete (GAB) showed visible precipitates of packets of $CaCO_3$ on the concrete's surface. The $CaCO_3$ crystal precipitates observed on the surface of bioconcrete was as a result of reaction involving calcium ions and bicarbonate, which was facilitated by the catalytic actions of CA. Similarly, distinct $CaCO_3$ precipitate was reported from the SEM micrographs of bioconcrete reported in the study of Maheswaran et al. (2014) and Ghelani et al. (2015).

5. Conclusion

Carbonic anhydrase (CA) producing bacteria were isolated from concrete soil sites and further screened for their CA production ability. The CA produced by *A. faecalis* (accession number MN847724.1) with enzyme activity of 0.0351 U/ml was applied in the biocalcification of concrete. The result showed a considerable improvement in strength, and with a percentage strength gain of 42.29 % as compared to the control with a percentage strength gain of 9.19 %. Results from the scanning electron micrograph revealed the presence of $CaCO_3$ precipitated in packets on the concrete's surfaces. The $CaCO_3$ fills the crevices of cements thereby strengthening the concrete. With the results obtained in this study, the CA could have huge potential applications in biocalcification of concrete.

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CRediT authorship contribution statement

Oluwafemi Adebayo Oyewole: Conceptualization, Supervision, Editing; **Naga Raju Maddela:** Methodology, Software; **Omeiza Haruna Ibrahim:** Data curation, Writing - original draft preparation; **Ifeoluwa Adebayo-Anwo:** Data curation, Investigation; **Taiye Elisha Adejumo:** Reviewing and Editing; **Emmanuel Oche Agbese:** Software, Validation; **Evans Chidi Egwim:** Supervision, Experimental design; **Ram Prasad:** Supervision, Writing - reviewing and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biteb.2023.101434.

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