BIOCALCIFICATION OF CONCRETE USING CARBONIC ANYHDRASE PRODUCED BY SOIL BACTERIAL ISOLATES

ABSTRACT

Two (2) bacterial isolates obtained from Garima construction sites were named GARIMA (A) and (B), and code-named GA (A) and GA (B) respectively. They were screened for their carbonic anhydrase (CA) producing ability. Isolates GA (A) and GA (B) showed positive reaction to para-nitrophenylacetate (pNPA), producing yellow and peach/orange coloured colonies respectively. The isolates were used to produce the crude CA and the mean enzyme activity with standard error of mean for the CA from isolates GA (A) and GA (B) were 0.0321 ± 0.0012 and 0.0351 ± 0.0002 mmoles/mL^{-1}/sec^{-1} respectively, the GA (B) was subsequently used for the large scale production of the crude CA. Isolate GA (B) was identified as *Alcaligenes faecalis* subsp. *parafaecalis* strain G, using cultural, biochemical and molecular characterizations. Optimum substrate concentration for the CA from isolate GA (A) was 5 mM, with 50°C optimum temperature and an optimum pH of 8.5. While CA from *A. faecalis* subsp. *parafaecalis* Strain G had an optimum substrate concentration of 7 mM, optimum temperature of 50°C, and optimum pH of 9.5. The crude CA extract was used to reinforce concrete; the results showed an increase in crushing strength on days 7, 14 and 28 with mean crushing strength values of 11.54, 15.52 and 22.28 N/mm^2 respectively, and with a percentage strength gain of 48.29%. The scanning electron micrographs revealed distinctly visible precipitates of calcium carbonate crystals on the surfaces of the concrete treated with the crude CA. These results indicate that CA could have huge potential applications in the bio-calcification and healing of concrete.

CHAPTER ONE

1.0   INTRODUCTION

1.1 Background to the Study

A very important material composition for building infrastructure and civil construction that is relatively inexpensive, within reach and easy to cast is concrete; a term used to describe any materials that have its components of cement, fine aggregates of sand, coarse aggregates of pebbles or stones, and water (Khattra, *et al.,* 2016; Patil *et al.,* 2016; Viduthalai *et al.,* 2018). The materials compositions of concrete are such that are able to withstand compressive weight to some extent, until the applied weight is greater that the resistance limit, thus causing a reduction in strength by forming cracks (Viduthalai *et al.,* 2018). The lack of durability in concrete is oftentimes connected to increased permeability, often as a result of the increasing permeability matrix or availability of cracks (De Belie and Wang, 2016). Concrete is sensitive to crack formation, thus endangering its durability, and often requiring repairs and of course attracting more cost. The formation of crack results from its lack of enough tensile strength fortification, reason for the addition of steel to concrete in order to accommodate the stretched weight (Van Tittelboom *et al.*, 2010).

When the tensile stresses are high on concrete, it causes deformation as a result of amongst others, silica reaction, gradients in the temperature and corrosion of the reinforcements. The development of small concrete cracks create an avenue for the passage of liquids and gases through them, damaging the concrete and corroding also the reinforcements, therefore leading to structural failure. Implying that the formation of cracks in concrete is a fundamental cause of structural failure, and a delay and inaccurate response to the cracks could cause further enlargement and additional repair costs (Bhavana, 2017; Neha *et al.,* 2018).

The occurrence of cracks in concrete is as a result of several processes which include tensile and mechanical compressive forces, shrinkages (Gandhimathi *et al.,* 2015). Micro-cracks in concrete, over time accelerate its deterioration as it allows permeation of deteriorating elements such as carbon (IV) oxide (CO_2), sulphates and chloride ions into the concrete body. And once the permeation of these elements is repetitive, it causes an expansion in the cracks width, leading to acceleration in the concrete deterioration (Choi *et al.,* 2016). Adequate attention must be focused on preventing the aforementioned process affecting concrete durability (Jokyani and Chouhan, 2018).

The conventional repair mechanisms for compromised concrete relies on the process of matching materials of dissimilar characteristics such as a composition of inorganic and organic calcium hydrosilicate and epoxies of petroleum origin respectively. A viable alternative to this approach is the application of bacteria and/or their metabolic products in concrete strengthening and repairs (Rahbar *et al.,* 2021). The method of concrete strengthening to heal these cracks has been employed in a quest to overcome these challenges associated with concrete cracking, in a technique called Bio-calcification or Microbially Induced Calcium Carbonate Precipitation (MICCP), involving a selective microbiologically mediated filling process whereby calcium carbonate ($CaCO_3$) is precipitated, and acting as a microbial sealant is encouraged by metabolic activities of the microorganisms, resulting in the reduction of the concentration of the openings and an improvement of the concrete's serviceability, longevity and compressive strength (Parnnika and Das, 2013; Gandhimathi *et al.,* 2015). An array of complex biochemical processes is involved in MICCP, which includes the precipitation of calcium carbonate ($CaCO_3$) by the action of the enzyme carbonic anhydrase (Varenyam *et al.,* 2010).

Also, these greenhouse gases' (GHGs) concentration in the atmosphere has assume an alarming dimension caused primarily from the activities of humans, which has led the

global campaign geared towards restoring the integrity of the climate. The greenhouse gases include but not limited to carbon iv) oxide (CO_2), methane and chlorofluorocarbons with CO_2 being the most prominent GHG. CO_2 arises from the combustion of fossil fuels and also from the processes of industrial activities such as the production of cement (Christopher *et al.* 2013).

The ubiquitous distribution of microorganisms makes them huge reservoirs of biochemically important enzymes and important candidates in enzyme production. Microbial enzymes are naturally robust and highly thermo- and pH- stable, and also highly multi-functional, making them ideal candidates for a wide range processes of biotechnology (Thapa *et al.,* 2019). Carbonic anhydrase (CA) is a metalloenzyme containing zinc, and that is able to catalyse the reversible hydration reaction of CO_2 (CO_2 + H_2O ⇔ HCO_3^- + H^+). Therefore, CA has the potential to speed up the process of precipitation of calcite under favorable conditions (Zhang *et al.,* 2011). Eventually, this acts as a sealant to the formed fissures and cracks in concrete by self-assembling on concrete surfaces and inside the cracks thus producing crystal substances that are stable and have these cracks filled with their solid precipitates. CA in the presence of calcium can be utilized to form calcium carbonate rapidly, and in addition the calcium carbonate produced has materials similarities with the concrete mechanical characteristics, thus creating a final product that is almost non-differentiable. Also atmospheric carbon (iv) oxide is sequestrated by the catalytic reaction of CA precipitation of calcium carbonate, and also its decomposition is not offensive or threatening to the lives of human. Although most of the CA are dependent on or contain zinc (Zn^{2+}), there have been reports of some cadmium (Cd^{2+}) and Iron/Ferrous (Fe^{2+}) containing CAs (Tomazett *et al.,* 2016; Rahbar *et al.,* 2021).

This biological technique can be employed to enhance the compressive strength and stiffness of concretes with cracks. MICCP or MECR can take place within or out of the cell of the bacteria, more so a shift away in concrete, with the metabolic activities causing the precipitation of minerals as a result of the changes in solution chemistry (Patil *et al.,* 2016). Some bacteria that can carry out such calcification function include *Bacillus sphaericus*, *B. megaterium*, which generally are regarded to as safe bacteria as they are non-pathogenic, and which apart from their calcification ability, thrive in a pH of 9.0 optimum (surviving in harsh environmental conditions as the high pH support the activities of the bacteria), are spore formers, affording them stay for a longer time in cement and concrete as capsules and also the secretion of exoploysaccharide substances that aids concrete adhesion (Acuna *et al.,* 2018; Whitaker *et al.,* 2018; Agereh *et al.,* 2019).

Carbonic anhydrase produced by most calcifying bacteria have been reported by Bansal *et al.* (2016) to have greater potential as a biocatalyst for $CaCO_3$ precipitation and formation from carbon (iv) oxide hydration, in the presence of a calcium source. Application of MICCP by exploring the potentials of carbonic anhydrase is an environmentally friendly approach as it does not cause the depletion of the resources of nature and also does not result in the release of harmful substances to the environment, prompting the proposal for its use in the construction industry as it helps in achieving a denser and stronger concrete (Satinder *et al.,* 2017). This research therefore aims at biocalcifying concrete using the carbonic anhydrase produced by isolated soil bacterial isolates.

1.2 Statement of Research Problem

Concrete is a major player in the civil construction industry, and it is being bedeviled by cracks formation that actually threatens concrete durability as the cracks allows for the permeation of liquids and gaseous substances into the spaces created by the cracks. This exposes the concrete reinforcements to environmental stress, often leading to corrosion of these reinforcements and structural failure subsequently which could come in the form of building, bridge collapse, pavements caving in (Van Tittelboom and De Belie, 2010; Patil *et al.,* 2016). The aforementioned can be tackled by constant maintenance and repairs. However, some cracks called micro-cracks are not easily noticeable, and could grow into bigger cracks that will eventually lead to structure collapse.

Odeyemi *et al.* (2019) while studying the trends of building collapses from 2009-2019, reported an alarming rate of cases of collapsed buildings in Nigeria. These spates of building collapse can be likened to the cracks on concrete infrastructure. Initially, when micro cracks are formed in concrete, they create huge damage to the ability of the concrete to be serviceable resulting in maintenance cost in the long run.

In addition, the cement industry is a critical player in the consumption of fossil fuels, contributing considerably to global carbon emissions. The production of cement releases carbon (IV) oxide (CO_2), a greenhouse gas into the atmosphere that causes the depletion of the ozone layer and ultimately global warming (Ali *et al*., 2015).

The methods that are conventionally employed for concretes crack repairs involve treatment with chemical adhesives or sealants. Conventional concrete cracks repairing methods such as the treatments with chemical sealants or adhesives for the prevention of the widening of the cracks are generally environmentally unfriendly and of high cost implications, and in addition there is also the issue of incompatibility with concrete in terms of the materials.

1.3 Justification for the Study

Considering the alarming rate of concrete structural failure arising from cracks, and the attendant monetary and human costs, a successful use of bacteria and/or their products for remediation of this menace is a great step towards having a revolutionary technology of using microbial products in civil construction and concrete strengthening. In addition, the race for the adoption of green technologies that are environmentally friendly is fast dominating global center stage, and the application of carbonic anhydrase (CA) which itself is a bacterial product will relatively help reduce green-house gas emission as it will sequestrate CO_2 and in the presence of calcium produces $CaCO_3$, which therefore calcify the concrete. Carbonic anhydrase is of great importance in this regard, as it is able to sequester CO_2 in a series of biochemical reactions to produce $CaCO_3$ (Bond *et al.,* 2001; Kanbar, 2008).

The use of microbial approach to concrete crack repair and healing has gained prominence and growing acceptance recently and has been reported to offer a better alternative, as it involves the application of microorganisms and/or their products for the treatment of concrete. This method is not only environmentally friendly; it also confers on the concrete the ability to self-heal on its own (Joshi *et al.,* 2017). This is a process known as bio-calcification or microbially induced calcium carbonate precipitation (MICCP); a highly effective technological process that has been applied in repairing or remediating concrete cracks, improving the strength of concretes, soil consolidation and even in restoring monuments made of stones. This process is eco-friendly, and very cost effective.

Microorganisms can actually produce metabolites that can precipitate $CaCO_3$ to fill in these cracks and sealing them, thus affording self-healing of concretes and reducing the permeation of liquid and gases into the crevices when formed and of course, therefore saving repair and maintenance cost while improving the durability of the concrete

(Tambunan *et al.,* 2019; Rahbar *et al.,* 2021). This research could be revolutionary, as it could help in strengthening concrete by way of bio-calcification, which could be applied in roads, bridges and pavements construction, and also housing infrastructure.

1.4 Aim and Objectives of Study

This research was aimed at biocalcifying concrete using carbonic anhydrase produced from soil bacteria isolates. The objectives of this study were to:

    i.   isolate and screen bacteria from soil of selected construction sites for carbonic anhydrase (CA) production.

    ii.   identify the bacteria with the higher potential for the production of carbonic anhydrase.

    iii.   determine carbonic anhydrase activity and optimize the parameters for carbonic anhydrase activity.

    iv.   determine the effect of the carbonic anhydrase on the bio-calcification of concrete.

CHAPTER TWO

2.0.   LITERATURE REVIEW

2.1 Concrete

A very key importance to global economy is structural durability and lasting performance, and with concrete being a key component for the provision of global infrastructures with relative durability and cost effectiveness when compared to other construction materials, there is need to ensure it is of a better quality to reduce structural failure and collapse. The rate of deterioration experienced by concrete infrastructures buildings, bridges is at an alarming rate, and it is even worse in structure that are in contact with hazardous chemicals such as offshore platforms and concrete pipes and tanks that serve as transport and holding containers for these chemicals (Zai and Murthy, 2015; Castro-Alonso *et al.,* 2019).

Concrete is a material of considerable strength used for building and construction purposes all over the world. Castro-Alonso *et al.* (2019) reported that over ten billion tons of concrete are utilized globally every year, and with a prediction that by the year 2050 this figure could jump to over sixteen billion tons. The authors further reported that this increased concrete utilization is a testament to the fact that they are hugely affected by a variety of factors ranging from physical, chemical and biological; in the form of variations in temperature, natural disasters, corrosion caused by exposure to chemical substances that are aggressively corrosive, and bio-corrosion caused by microbial activity.

In addition, these factors according to the authors cause the formation of micro-cracks ultimately affecting the strength and durability of the concrete.

It is made up of added components of water, coarse and fine aggregates in a matrix of cement occupying the crevices created in these aggregates and sticking them together. The utilization of concrete dates back to the pre-Roman Empire, and has found wide usage

in ancient history in nations like Egypt and Greece. And after Joseph Aspdin in 1824 patented the Portland cement, it has since been used for buildings and infrastructure construction world over (Bamigboye *et al*, 2015). With good fire resistance and compressive strength, but with relatively low tensile strength when compared to the compressive strength, this has necessitated more studies towards increasing concrete strength. One of those is the use of microorganisms and/or their products for concrete strengthening (Bamigboye *et al*, 2015; Ede and Aina, 2015).

Concrete is widely applied for civil construction works globally as a result of its high performance properties and prolonged lifespan. A huge chunk of resources that are non-renewable go into civil construction, contributing largely to the atmospheric carbon (iv) oxide (CO_2) build-up. More so, the process of concrete production require high energy input when one put into consideration the mining of the raw materials, transporting and processing, contributing about 10% of the global atmospheric CO_2 emission. One of the shortcomings of concrete is that it is prone to cracking upon experiencing tension, and an expansion of these micro cracks could create huge cracks that could further allow permeation of liquid and gaseous substances into the crevices. Often leading to the hitherto internal steel used to reinforce the concrete to be exposed to corrosion, and also a decline in its lifespan and its functional properties (Sujatha *et al.,* 2014; Jokyani and Chouhan, 2018; Seifan and Berenjian, 2018). When the steel reinforcements are corroded, it compromises the structure of the concrete, in terms of mechanical strength. In addition, this corrosion distorts the aesthetics of the concrete (Sujatha *et al.,* 2014).

The most prominent defect usually noticed in concrete is cracks, which normally occur as a result of shrinkage during drying or curing, freeze-thaw reactions, mechanical compressive and tensile forces, or due to the imbalance in the proportion of the concrete

compositions. Cracks reduce the tensile strength of concrete and its durability, by creating a pathway for external agents such as water, other liquid substances like chlorides, and some harmful gaseous substances, that when they make contacts with the concrete reinforcements such as iron and steel rods, corrodes them thereby weakening them. Subsequently leading to failure and collapse of the concrete structure (Kashyap and Radhakrishna, 2013; Gandhimathi *et al.,* 2015).

Neha *et al.* (2018) reported that, cracking of concrete is an inherent property because even after ensuring all recommended safety procedures in the design of the concrete mix and in the use of good concrete materials, yet cracks do occur most times, as a result of several factors such as the escape of trapped air which eventually create spaces in between the concrete particles. The authors further reported that the permeation of water and other gaseous substances through these crevices that further exposes the concrete reinforcements to unfavorable conditions, thus affecting the integrity of concrete.

These cracks are often repaired using the conventional repair mechanism such as the use of chemical adhesives and petroleum epoxies, which are not without some shortcomings such as their health implications, environmental unfriendliness and thermal expansion coefficients that are at variance with the concrete. This has led to the adoption of a more viable health and environmental friendly option using bacterial species and/or their products called Microbially Induced Calcium Carbonate Precipitation (MICCP) (Kashyap and Radhakrishna, 2013).

Increased rate of global concrete usage stems from the fact that they are susceptible to attack from several factors such as varying temperature, low quality composition, actions of microorganisms, corrosion, and natural catastrophes such as hurricanes and flooding. As a result of the aforementioned factors, concrete are prone to micro cracking which

ultimately grow bigger which allows the ingress of corrosive liquids and gases, thus having a huge impact on the compressive strength and also the flexural strength of the concrete, causing a reduction in its durability and high maintenance and repair costs. Researches into the exploitation of the metabolic processes of bacteria and some other microorganisms have grown in the last couple of decades to proffer solutions to the problems bedeviling concrete using an environmentally friendly approach, a process known as microbially induced calcium carbonate precipitation (MICCP). The products of this process have been collectively referred to as bio-concrete, which is conceptualized on the use of bacteria and/or their products to induce calcite precipitation on the concrete (Castro-Alonso *et al.,* 2019).

2.1.1 Bio-concrete

An emerging technology in the world today, with huge potentials of sustainability and reduction of the harmful environmental impacts of the emitted atmospheric CO_2 while also addressing the issues associated with concrete cracks is the bio-concrete. This is achieved by the process of bio-calcification or MICCP involving a series of biochemical reactions carried out by enzymes such as carbonic anhydrase, coordinating a series of reaction that results in bio-calcification (Castro-Alonso *et al.,* 2019).

Advances in technology has ushered-in the development of a variety of concrete types including the conventional concrete, fibre concrete, fly-ash concrete, and the environmentally friendly type called bio-concrete. The latter has been given more research attention owing to its huge strength quality and its ability to self-remediate (or self-heal), thus improving its mechanical properties such as the compressive strength of the bio-concrete. The compressive strength is one of the major considerations in concretes production. The use of bacteria and/or their products to aid the filling and sealing of the cracks in, and strengthening of, concretes accelerates the calcium carbonate or calcite

precipitation by the diffusion, through the wet concrete, of CO_2, forming in the intermediate bicarbonate ion (Alshalif *et al*., 2018).

This increases the compressive and split-tensile strengths and in addition reduces the water penetration of the concrete. The chief enzymes that drive this process are the urease and carbonic anhydrase. The urease produces ammonia and CO_2, helping to increase the pH and making the medium alkaline. The carbonic anhydrase sequester the CO_2 and precipitates the available calcium ion together with bicarbonate ion to produce calcium carbonate. This is the process of bio-calcification and the resulting concrete is referred to as bio-concrete (Alshalif *et al*., 2018). The production of bio-concrete involves the incorporation of bacteria and/or their products to induce biomineralization (formation of minerals), thereby inducing the concrete to self-heal and also causing an improved strength and durability (Castro-Alonso *et al.,* 2019).

2.2 Biomineralization

The process of biomineralization involves the biological mineral synthesis by microorganisms, mediated by enzymes. The processes naturally take place in different environments, involving different microorganisms with different metabolic pathways. Inorganic compounds such as the oxides of phosphorus, carbon, silicon, sulphur, and also the inorganic anion of sulphur, in combination with various cations of calcium, ferrous, magnesium, and manganese oxide form biominerals by the action of microorganism. This process also includes macromolecules of organic origin like the proteoglycans, proteins, polysaccharides and glycoproteins that function as a form of skeletal support (Ghosh *et al.,* 2009; Sarayu *et al.,* 2014). There are three major mechanisms of the biomineralization process namely: Biologically Controlled Mineralization (BCM) (as presented in Figure 2.1), Biologically Induced Mineralization (BIM) (Figure 2.2 depicts), and Biologically Mediated Mineralization (BMM) (Figure 2.3) (Dupraz *et al.,* 2009; Achal *et al.,* 2015).

In the mechanism of biologically controlled mineralization (BCM), the nucleus, components, concentration, and structure of the biominerals are controlled by the metabolic activity of the participating microorganisms. This mechanism could occur outside of the cells (BCMe), inside the cells (BCMin), and an in between cells (BCMint) process *via* the contribution of exopolysaccharides (EPS) or vesicles of organic macromolecules (Weiner and Dove, 2003).
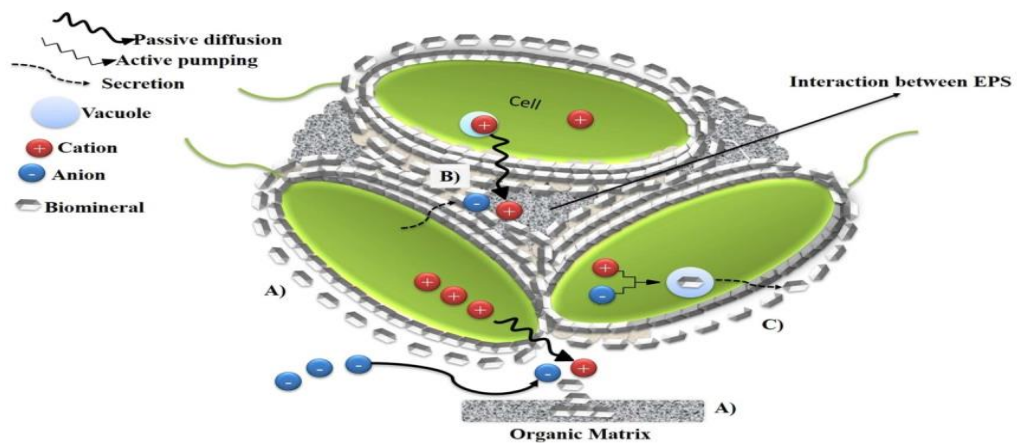

Figure 2.1: Schematic diagram of Biologically Controlled Mineralization (BCM). Source: Weiner and Dove (2003).

In the course of the biologically induced mineralization (BIM), the precipitation of minerals is achieved indirectly *via* the collaborations between the end-products of microbial metabolites and the ions present in the environment (Figure 2.2). Nonetheless, in this process, there is a restriction of involvement of microbial cells in how the minerals are constituted, localized and nucleated. Their wide range describes the minerals produced by biologically induced mineralization (BIM) in size of morphology, particulates, and poor crystallization (Weiner and Dove, 2003).
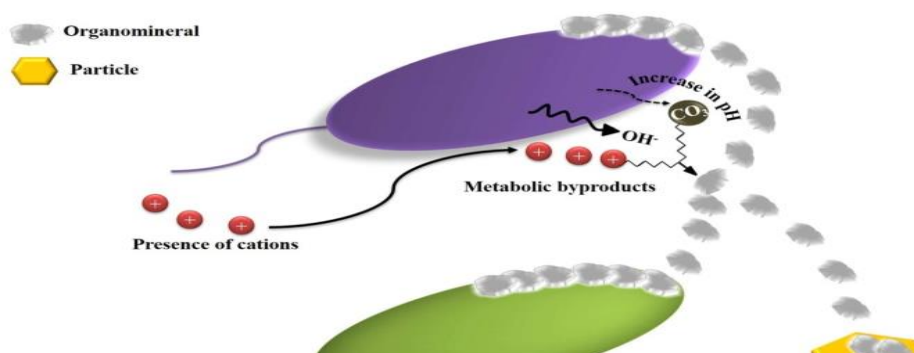
Figure 2.2: Schematic representation of BIM-induced biomineral precipitation due to the interaction of metabolites of microorganisms with existing inorganic compounds in the environment.

Source: Weiner and Dove (2003).

Mineral formation in the biologically mediated mineralization (BMM) process occurs as a result of an interface between inorganic compounds and an organic matrix without necessarily involving biological activities outside or inside the cells (Figure 2.3). Dupraz *et al*. (2009) reported the exclusion of BIM and BMM from the processes of biomineralization and introduced instead the term organo-mineralization for these biological processes. The most prominent biominerals and organo-minerals are made up of calcium as a significant ion, as a result of its (calcium) participation in the numerous fundamental processes in the organisms' cellular metabolic activities. Minerals containing calcium encompass about half of the formed total biominerals and organo-minerals.

$CaCO_3$ is one of earth's most abundant minerals, including making up about 4% in the earth's crusts total weight. Calcium carbonates deposition commonly occur in the oceans, sediments, freshwater, soil, and other environments known as microbial calcium carbonate (MCP) deposition. The deposition process can be active or passive; active BIM is also called microbial induced $CaCO_3$ precipitation (MICCP) and passive BMM, is a

product of the interaction between the exopolysaccharides (organic matrix) and the ions of calcium with no need for an activity that is directly biological. In the process of BMM, certain functional groups like the carboxylic acid (R-COOH), hydroxyl (R-OH), amino (R-NH$_2$), sulfate (RO-SO$_3$H), and sulfhydryl (-SH) can be raised from pH. This creates an overall negative charge on the peptide-forming bonds that surrounds the cells, thereby encouraging their strong attachment to the metal ions. Figure 3 illustrates the significance of the organic matrices in the structure and scientific study (mineralogy) of CaCO$_3$ (Dupraz *et al.,* 2009; Sarayu *et al.,* 2014).
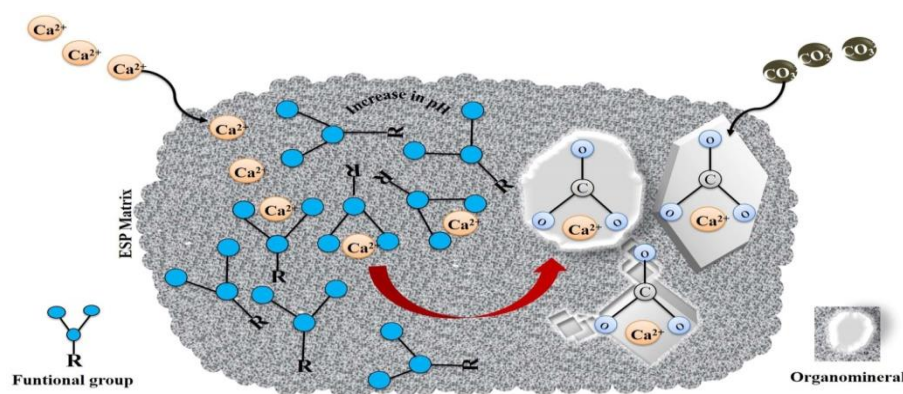

Figure 2.3: Schematic illustration of Biologically Mediated Mineralization (BMM). Source: Dupraz *et al.* (2009)

2.3 Carbonic Anhydrases

The application of enzymes for biotechnological purposes is going through a gradual phase of development, and their functional characteristics of selectivity, efficiency and high catalytic rate make them useful candidates in a whole range of environmental-friendly industrial approaches (Binod *et al*., 2013). Microbial enzyme sources are

employed for these various industrial processes owing to their relative stability and many distinct characteristics that are quite different from other enzyme sources. In addition, their physiological and physic-chemical characteristics can be readily manipulated and regulated, obtained with little cost by extracellular extraction and can also be produced quantitatively (Binod *et al*., 2013).

Microbial enzymes such as the carbonic anhydrases (CAs, EC 4.2.1.1) have found useful application in diverse areas of technology. These metallo-enzyme groups that contain zinc and can perform the catalysis of converting carbon (iv) oxide to bicarbonate thereby releasing in the process a proton. This process is reversible (Imtaiyaz *et al.,* 2013). These enzymes have active sites made up of Zn^{2+}, and this is organized and held together by three residues of histidine and a complex of hydroxide ion and a molecule of water. This inter-conversion process involving carbon (iv) oxide and the ion of bicarbonate is catalysed by CA, transferring hydrogen ions through the CA active sites and the buffer that surrounds it, leading to an alteration of the pH upon the progression of the reaction process to a state of equilibrium, thus increasing it from 8.2 to 8.4. And with the availability of calcium ions, calcite or $CaCO_3$ is formed, with CA providing the needed platform with the available appropriate cation and pH for the calcite formation (Komala and Khun, 2014). Carbonic anhydrases can function as a hydratase, an esterase and in the carbonate formation. This process of CO_2 hydration is identified as a rate determinant in the entire CaCO3 precipitation reaction aided by the availability of calcium ions (Ca^{2+}) (Muller *et al.,* 2013).

Carbonic anhydrases (CAs) are encoded by bacteria, and these CAs belong to the three classes of α-, β- and γ-CAs. Their ability to convert CO_2 generated during metabolic process into products that are soluble such as the bicarbonate and protons, makes them significant in a wide range of physiological processes associated with the secretion of

electrolytes, pH homoeostasis, photosynthesis and other biosynthetic processes involving CO_2 and bicarbonate. The critical involvement of CAs in bacterial metabolism, explains the reason why they are widely found in both Gram-negative and Gram-positive bacteria. In addition to their highly effective role in the hydration process of converting CO_2 to bicarbonate and protons (Vulloa *et al*., 2017). The hydration step is the rate determinant in the process of conversion of CO_2 to bicarbonate ion, thus making the significance of CA highly appreciated when the reaction is presented with limited carbon concentration during the calcification process. Carbonic anhydrase plays a very crucial part during the formation of eggshells in birds, while also having huge contribution in the formation of otoliths in fishes. Also in invertebrates such as molluscs, calcerous sponges, scleractinian corals and octocorallians, CAs play a critical role in their calcification (Tambutte *et al.,* 2007).

Cells and tissues when they partake in an array of critical biological metabolism including respiration, ureagenesis, ions and CO_2 transport, calcification and photosynthesis, produce CAs in the process. Carbonic anhydrases have different isozymes, and about 16 of these have been so far described, each differing in their enzymatic activity. In addition, they are encoded by unique genetic varieties of four variations and ubiquitous throughout the phylogenetic tree (Ores *et al*., 2012; Dutta *et al.,* 2015). They basically are significant to biological systems, and have found useful industrial applications. Carbonic anhydrases also are critical players of physiological and patho-physiological processes. In addition, they are also one of the enzymes produced by bacteria used for bio-calcification of concrete (Imtaiyaz *et al.,* 2013).

Carbonic anhydrases have crucial impact in the fixing of carbon in organisms like algae, cyanobacteria and plants during photosynthesis. For these organisms to produce biomass, the rate determining step is the CO_2 uptake which usually is in the form of bicarbonate.

The enzymes carbonic anhydrases are mostly zinc dependent metalloenzymes or metalloproteins that aid the catalysis of the reversible hydration of carbon (iv) oxide (CO_2), generating at the end of the reaction, proton and HCO_3^+. This process when it happens without the catalytic effect of CA is quite slow (Dutta *et al.* 2015; Ibrahim *et al.,* 2016).

These enzymes have a widespread occurrence or ubiquitous distribution in all life kingdoms, and are classified into three (3) namely alpha (α), beta (β) and gamma (γ) carbonic anhydrases, all existing in different structures and are also not related in terms of evolution (Christopher *et al.,* 2013). The α-classes of carbonic anhydrases are readily obtainable throughout the kingdom Animalia, and also found in the periplasm of the bacteria *Chlamydomonas reinhardtii*, some *Neisseria* sp. such as *Neisseria gonorrhea*. The β-classes of carbonic anhydrases on the other hand are mostly found in the monocotyledonous or dicotyledonous plants. The *Archeabacterium* called *Methanosarcina thermophila,* provided the first platform for the isolation and characterization of the γ class of carbonic anhydrase. The α-class of the CA has high activity in the monomeric state, while the β-classes are known to form dimers, which sometimes transform into tetramers or octamers. The γ-classes on the other-hand form structurally homotrimeric. Carbonic anhydrases are capable of utilizing the same mechanism of catalytic action, even with their variation in structural composition (Christopher *et al.,* 2013; Pander *et al.,* 2019).

As a result of their catalytic efficiency, low production cost, and reusability, carbonic anhydrase have gained recent interest for application in biocalcification and the sequestration of atmospheric carbon. This process of converting CO_2 into calcite, a product that is environmentally friendly, is gradually gaining attention recently, as it can be applied in concrete strengthening, filling and sealing to overcome the challenges of

cracking in concrete. The calcite is a major component of the shells of organisms in the marine habitat, and can be produced by the reaction of the catalytic product of carbonic anhydrase and a calcium source (for example bicarbonate and calcium chloride) (Christopher *et al.,* 2013).

2.4 Biocalcification or Microbially Induced Calcium Carbonate Precipitation (MICCP)

Biocalcification otherwise referred to as microbially induced calcium carbonate precipitation (MICCP), is a biological process involving a selective microbiologically mediated filling or strengthening of concrete in which calcium carbonates ($CaCO_3$) are precipitated, acting as a microbial sealant and therefore improving the strength properties of concrete. This process is encouraged by the metabolic activities of microorganisms, resulting in the reduction of concentration and intensity of the openings, and an improvement of durability and compressive strength of the concrete (Parnnika and Das, 2013; Gandhimathi *et al.,* 2015). In addition, an array of complex biochemical processes is involved in the MICCP, which includes the precipitation of $CaCO_3$ by the action of the CA (Varenyam *et al.,* 2010).

In MICCP, the formation of $CaCO_3$ minerals results from the interaction among different metabolites. There was a report that MICCP occurs only by extracellular agents. Several studies have shown the precipitation of intracellular calcium carbonate in cyanobacteria (Cam *et al.,* 2018; Benzerara *et al.,* 2021). Head *et al*. (2000) in their study found and reported that agents operating intracellularly aid the precipitation of calcite crystals from chromium oxalate. The authors pointed out that intracellular calcium carbonate crystals absorbed more than 70% of the cells in the total number of cells and are membrane bounded by membranes without bubbling. Xu *et al.* (2019) in a study reported the

deposition of virus-induced cyanobacterial cell lysis of calcite and aragonite, and showed that this new mechanism prolongs the process of biomineralization of calcium carbonate.

MICCP has been reported in many biotechnology applications, such as in soil repairs and consolidation, concrete repairs, healing and strengthening, carbon dioxide sequestration and in the bioremediation of heavy metals contaminated water, metalloids and cations (Ca^{2+}) (Li *et al.,* 2018, Kang *et al.,* 2014; Wang *et al*. 2014; Yadav *et al.,* 2014; Okyay and Rodrigues, 2015; Siddique *et al*, 2016; Chen *et al.,* 2016).

2.4.1 Application of carbonic anhydrase in microbially induced calcium carbonate precipitation (MICCP)

One mineral of biological origin making up the inorganic supporting frameworks of skeleton related components in some organisms such as the coccolithophores, which are unicellular algae and non-metazoan, is calcium carbonate ($CaCO_3$). Evolutionarily, the calcareous sponges are among the earliest known metazoans found to have devised $CaCO_3$ for their skeletal support. $CaCO_3$ in most cases are acquired through an uptake from the surrounding aqueous medium by a mechanisms of membrane transport that is specific and best described by the Michaelis-Menten constant of around 50 mM, where 50% precipitation of CaCO3 is recorded (Muller *et al.,* 2013).

In biological systems such as microorganisms, calcium carbonate is precipitated as calcite, aragonite or vaterite in some cases. This precipitation can be formed intracellularly or extracellularly, and the variation depends upon the organisms carrying out the process. For calcium carbonated ($CaCO_3$) to be precipitated, three (3) critical steps are involved which include a supersaturated solution, followed by crystal formation otherwise called nucleation which usually starts from the saturated point and lastly, the proliferation of these crystals spontaneously. The abundance of $CaCO_3$ makes it

promising as an alkaline producer for application in biotechnology (Salek *et al.,* 2014; Anbu *et al*., 2016).

Biocalcification or MICCP therefore is the process of calcite formation from solution that has been supersaturated as a result of the availability of microorganisms and of course, their metabolic activities. In this process, the participating microorganisms have their products of metabolism such as bicarbonate ions (CO_3^{2-}) reacting with cations such as (Ca^{2+}) present in the surrounding environment to produce CaCO3 in the form of precipitates (Anbu *et al.,* 2016).

In environmental studies and some processes of engineering, the use of microorganisms and their metabolic products is fast gaining grounds as researches are ongoing to better the technology. That this process of microbial application is suitable for environmental purposes is not in doubt, as a number of researches have proven that microbes and their product(s) can be applied for the bioremediation of wastewater and soils contaminated with hydrocarbons. Some of the microorganisms found to be applicable in environmental remediation precipitate calcium carbonate ($CaCO_3$), sequestration of atmospheric CO_2, and these processes have had huge impact in geology and of course, the carbon cycle (Navdeep *et al.,* 2018). Researches in MICCP over the last 10 years has revealed the positive outcomes of the application of this technology, coming against the backdrop of its earlier use in the remediation of stones of historical significance. This ecofriendly approach has found huge applications in concrete and by extension in the civil and construction field of engineering (Wang *et al.,* 2016).

Similarly in civil engineering, the precipitation of carbonate by microorganisms especially bacteria have been of growing interest as it can be employed for bio-cementation, made possible by the crystallization of $CaCO_3$. The group of bacteria responsible for carrying out bio-cementation are called calcifying bacteria and they

include *Bacillus megaterium, B. sphaericus, Sporacinia pasteureli*, *Lysinibacillus fusinformis,* and they have been largely applied in the consolidation of sand, repair of monuments made up of lime stones, and filling and repair of concrete cracks and concrete enhancement. This is the process of microbial induced calcium carbonate precipitation or MICCP, also referred to as bio-calcification (Navdeep *et al.,* 2018).

Dhami *et al.* (2013) reported that successful researches have been conducted on the use MICCP to improve the concrete strength, stating that bacteria identified as *Bacillus pasteurii* and *B. sphaericus* capable of producing carbonic anhydrase and urease were employed and found to have had great impacts on the strength properties of the concrete samples and provided resistance in concrete against shrinkage from drying and improving the compactness thereby reducing the permeation. Similarly, the authors also reported this organism to have remedied the cracks in concrete and restored the compressive strength of concrete by about 84% when compared to the untreated control. In addition, Lee *et al.* (2015) reported also that applying microorganisms and/or their products have led to an increased compressive strength of concrete upon curing in a liquid medium, as this causes a hydration of the concrete thus having a huge effect on the compressive strength.

MICCP is a procedure whereby microorganisms are able to formulate a localized micro-medium having all the essential compositions allowing for calcite precipitation. It is primarily directed by factors such as the calcium concentration, level of the dissolved inorganic carbon, presence of nucleation sites and of course pH. The process requires adequate ions of calcium and carbonate in order to allow for the product of ion activity go beyond the solubility constant (Dhami *et al.,* 2013).

Researchers have shown that MICCP produces three different forms of $CaCO_3$ as anhydrous polymorphs of calcite, vaterite and aragonite; as hydrated crystalline forms of

ikaite and monohydrocalcite; and as amorphous phases. But in all of the above, the most prominent polymorphs of MICCP are calcite and valerite. Bacteria primarily dictate MICCP by their ability to utilize their physiological processes create a medium that is alkaline and favourable for the process (Dhami *et al.,* 2013).

This technology is emerging and has generated huge interests from researchers as it holds interesting promises for the prevention of cracks in concrete and for remediating and restoring concretes from cracks through a self-healing process (Bansal *et al.,* 2016). Leading to the concept of developing bio-concrete, which has been found in most cases to have had enhanced strength and are very durable. It has also been observed that bio-concretes have a deposit of calcite upon examination with scanning electron microscopy (SEM) (Castro-Alonso *et al.,* 2018). One of the most important materials in concrete is $CaCO_3$, normally derived from the concrete cement matrix components, and some bacteria have been found to induce the production of this vital material through their metabolic processes (Sefian and Berenjian, 2018).

Microbial or bacterial induced calcium carbonate precipitation in recent times has been proposed as an environmentally friendly approach to concrete crack remediation and prevention, and also applied for the protection of decayed ornamental stone (Vahabi *et al.,* 2018). The use of MICCP in engineering fields like civil or construction engineering, environmental and geological engineering has assumed a much sought after ecological friendly approach (Kim *et al.,* 2012). This approach explores the ability of the microorganisms or bacteria in use to produce precipitates of calcium carbonate with compatibility on the concrete or limestone. In sharp contrast to any other treatment often used in crack repairs such as lime-water treatment, the product of carbonated cementious compounds formed by MICCP has been reported to be highly coherent. The technique of

MICCP is hinged upon some common microbial or bacterial processes of metabolism such as the hydrolysis of urea, photosynthesis, sulphate reduction and carbon sequestration (Vahabi *et al.,* 2018). In addition, Vahabi *et al.* (2018) reported from their study that the net negative charge on the cell surfaces of microorganism possess scavenging abilities on cations that are divalent such as Ca^{2+} and Mg^{2+}. This they achieve by fastening or attaching the cations onto the surfaces of their cells, thus microorganisms are desired crystal central sites for MICCP.

The process of $CaCO_3$ precipitation is one that can be controlled seamlessly to precipitate a higher amount of $CaCO_3$ within a little time frame. The surfaces of the microbial cells serve as a platform for the precipitation of $CaCO_3$ provided there are adequate amount of the reacting ions of Ca^{2+} and CO_3^{2-}. A number of bacterial species that are soil inhabitants and also found in natural minerals have been reported to have the ability to carry out the calcium carbonate precipitation, for example *Bacillus sphaericus, B. subtilis, Sporacinia pasteurelli* (Kashyap and Radhakrishna, 2013; Anbu *et al.,* 2016)*.*

Bacteria mostly reported to cause the precipitation of $CaCO_3$ are from the genus Bacillus, and have been engineered for MICCP. Some are used for cracks healing in concrete and they include *Bacillus sphaericus* and *Sporosarcina pasteurii* (formerly known as *B. pasteurii*). Others such as *Bacillus cohnii* and *Bacillus pseudifirmus* have been reportedly used for surface treatment of concrete. Samples from soil and sludge have been the major sources of these bacteria. As reported by Omoregie *et al.* (2016), some bacteria capable of carrying out MICCP were isolated at various locations of the Palestinian Gaza strip from samples such as surfaces of concrete, soil and sludge. Examples of these bacteria include *Bacillus licheniformis, B. cereus, B. subtilis, B. lequilensis, Psuedomonas apiaries, P. antarcticus, P. borealis,* and *P. cellulositropicus*. Dhami *et al.* (2013) also reported the isolation of bacteria with close relation to *Bacillus megaterium*, *B. cereus*, *B.*

subtilis and *Lysinibacillus fusiformis* from a district in India called Anantapur. The sources of these bacteria were samples from calcareous soil. Similarly, Sarayu *et al.* (2014) reported a number of bacteria that are capable of precipitating $CaCO_3$, which include *Pseudomonas putida, Desulfovibrio desulfuricans,* and *Homoeothrix crustaceans.*

The actions of MICCP are brought about by mechanisms that are physically dependent, and hinged on the precipitation of $CaCO_3$ in relation to the other particles involved. $CaCO_3$ has the ability to thrive and successfully coat grain surfaces, and they can also precipitate close to the particles involved. In addition, the crystals of $CaCO_3$ may also migrate into the empty and loose spaces and in the end bridging these spaces with support from the matrix (Nayanthara *et al.,* 2019). The process of calcite precipitation requires that calcium ions (Ca^{2+}) and carbonate (HCO_3^{2-}) are mobilized alongside a formation that permits the occurrence of crystals at a nucleus. Carbon (iv) oxide is fixed into bicarbonate ions, thus providing the carbonate. This is a process catalysed by Carbonic Anhydrase (Else *et al.,* 2003).

2.5 Microbial Metabolic Processes in MICCP

Microbial metabolism hugely drives the MICCP, and some major processes involved include the hydrolysis of urea, amino acids ammonification, denitrification, dissimilatory sulphate reduction and photosynthesis (Dupraz *et al.,* 2009), with urea hydrolysis being the less complicated of the processes (Achal *et al.,* 2011).

2.5.1   Urea hydrolysis

The sequence of urea hydrolysis is a complex reaction catalysed chiefly by two (2) enzymes; urease and carbonic anhydrase (EC 3.5.1.5 and EC 4.2.1.1 respectively). The process involves the hydrolysis by urease, one mole of urea to one mole of ammonia and

carbamate, and the spontaneous hydrolysis of carbamate to form one mole of ammonia and carbonic acid. The carbonic acid is further transformed into bicarbonate by the carbonic anhydrase, eventually producing two (2) moles of ammonium and hydroxide as a result of the hydrolysis of ammonia. Consequently, the reaction process causes an increased pH in the cell surrounding thus inducing the calcite precipitation in the presence of soluble calcium. When the conditions are unfavorable, the survival of the cell is achieved by permitting the influx and settling of calcium ions, which results in protons being excessively expelled from the cells. Subsequently, to make up for the proton loss, calcium is actively exported from the cells. A microenvironment low in proton concentration and highly concentrated with calcium is significantly suitable for carbonate ions secretion, while the cellular super-saturation with carbonate induces $CaCO_3$ precipitation on the cell surface (Van Tittelboom *et al.,* 2010; Achal *et al.,* 2015; Anbu *et al.,* 2016).

2.5.2 Ammonification of amino acids

This mechanism involves the production of carbon (iv) oxide and ammonia during the microbial metabolism of amino acids. Production of ammonium and hydroxide ions around the cells is as a result of the hydrolysis of ammonia, resulting in the supersaturation of the cellular surroundings. This mechanism has been reported to be employed by *Myxococcus xanthus* when growing in liquid and solid media, thus resulting in varying polyforms (Chekroun *et al.,* 2004; Jimenez-Lopez *et al.,* 2007). It has also been further reported by Turick and Berry, (2016) that this organism precipitates uranium as a meta-autunite, reputed to offer protection to concrete structures against the effect of radioactive wastes.

2.5.3 Denitrification

MICCP results from denitrification as a result of organic matter oxidation, employing nitrate as the final electron acceptor. The reaction gives off nitrogen dioxide, carbon (iv) oxide, and hydroxyl ion, with the bacteria creating a microenvironment that is alkaline due to the assimilation of H^+ in the presence of soluble ions of calcium (Zhu and Dittrich, 2016). Ersan *et al*. (2019) reported the incorporation of denitrifying bacteria and expanded particles of clay with immobilized *Pseudomonas aeruginosa* and *Diaphorobacter nitroreductase.* This study revealed that the addition closed the microcracks in the range of 200-250 μm size and a decreased permeability by 42% and 47%, respectively. Similarly, Ersan *et al*. (2018) implemented individual granules called "activated compact denitrifying core" (ACDC) and found the healing of cracks (>70%) larger than 400 μm. However, the process of denitrification is hindered by the accumulation of byproducts with toxic composition such as nitrite and nitrous oxide.

2.5.4 Dissimilatory sulfate reduction

In anaerobic environments rich in organic matter, the presence of calcium induces the discursive mineral precipitaion by sulphate-reducing bacteria (SRB), due to the dissimilatory sulphate reduction reaction. Studies have shown that *Desulfovibrio sp*. can cause the precipitation of $CaCO_3$ through sulphates removal from gypsum by combined mechanisms of dissolution, diffusion, and precipitation of $CaCO_3$. Ca^{2+} released by the dissolution of gypsum under a microenvironment with alkaline pH (caused by the removal of sulphide), reacts with carbon dioxide (CO_2), leading to the precipitation of $CaCO_3$ (Perito and Mastromei, 2011).

Alshalif *et al*. (2018) reported that an introduction of SRB in a matrix of concrete caused an increased compressive strength by 13% and decrease in water permeability

by 8.5%. Similarly, Tambunan *et al.* (2019) reported an increased compressive strength of 60.87% and flexural strength of 52.30% by addition of SRB isolated from domestic acidic water. However, O'Connell *et al.,* (2010) reported that this generation of hydrogen sulphide (H_2S) can cause concrete corrosion because of the reaction of H_2S with oxygen to form elemental sulphur or partially oxidized specie of sulphur, which are considered to be corrosion products of concrete surfaces. Few studies have reported an association of SRB and cyanobacteria have caused improvement in the precipitation of $CaCO_3$. Extracellular polymeric substances produced by cyanobacteria as diverse nucleation sites, affect the diffusion barrier and the mobility of calcium ions, and improve the kinetics of precipitation by SRB. This makes the consortium of SRB and cyanobacteria appear to be promising candidates for application in the technology of bioconcrete, although more studies are required on the combination of oxygen-producing cyanobacteria with anaerobic SRB. (Kim *et al.,* 2005; Dupraz *et al.,* 2009).

2.5.5 Photosynthesis

The possibility of MICCP *via* autotrophic processes, such as photosynthesis and the oxidation of methane, apart from the usual heterotrophic bacterial processes of metabolism, have also been reported. The main photosynthetic microorganisms capable of carrying out MICCP in the aquatic environment are the cyanobacteria and microalgae. Precipitation of $CaCO_3$ by photosynthetic microorganisms occurs as a result of the exchange between HCO_3^- and CO_3^{2-}; there is diffusion of the HCO_3^- through the cellular membrane and dissociates in the cell's cytosol into CO_2 and OH^-. This reaction is catalyzed by carbonic anhydrase (CA), which leads to an increasing pH resulting from the generation of OH^-, which alongside the presence of calcium ions in the microenvironment induces MICCP or biocalcification (Dhami *et al.,* 2013; Achal *et al.,*

2015). The application of photosynthetic microorganisms as agents of biocalcification of concrete however, can only be achieved with the exposure of the concrete structures to CO_2 and sunlight, the two of which are huge components for the photosynthesis process (Seifan and Berenjian, 2018).

2.5.6 Methane oxidation

The concentration of CO_2 in marine and freshwater sediments is driven chiefly by methane-oxidizing bacteria (MOB), which derives their energy from the oxidation of methane, under both aerobic and anoxic conditions. In conditions that are aerobic, the process begins with the transformation of methane to methanol by the activities of methane monooxygenases which utilise the available oxygen. In the cellular periplasm, methanol which serves as the source of carbon is converted into formate through a series of enzymatic reactions. Subsequently, the formate upon equilibrium with formic acid, the methane monooxygenase *via* oxidation converts the formic acid to CO_2 through the activity of formate dehydrogenase. The CO_2 produced transforms into CO_3^{2-}, and then precipitated into $CaCO_3$ in the presence of calcium ions present in the cellular surroundings (Ersan, 2019).

2.6 Factors Affecting MICCP

MICCP as a process is controlled by a variety of factors such as the availability and concentration of the reacting ions, pH, the dissolved inorganic carbon (DIC) concentration, size of the bacteria involved and the concentration of the cells, and the presence of nucleation sites (Dhami *et al.,* 2013; Anbu *et al*., 2016; Omoregie *et al.,* 2016).

2.6.1 Reactants concentration and availability

The MICCP, the surrounding medium of the bacteria must provide the necessary ions such as Ca^{2+}, because of the critical role it plays in calcification. Traditionally, microorganisms have negative charge on the surface of their cells, helping them to attract positively charged ions such as Ca^{2+} which become bound to their surfaces in a liquid medium. The bacteria involved in MICCP produce bicarbonates from their metabolic processes involving carbonic anhydrase, and when these bicarbonates are discharged they come in contact with the free Ca^{2+} already present in the surrounding medium to form calcium carbonate. This process apart from carbonate precipitation, also ensure the survival of the bacteria in the calcium-rich medium by fixing it to the bicarbonate being released into the surrounding (Sarayu *et al.,* 2014; Omoregie *et al.,* 2016).

2.6.2 pH

Another important factor in the biocalcification or MICCP process is the pH, as it dictates the pace of activity and of course the microbial survival. The precipitation of $CaCO_3$ is greatly dictated by pH owing to the fact that the participating enzymes will have increased enzymatic activities at certain ranges of pH. In addition, the products production is largely dependent on the pH of the medium. The transformation of HCO_3^- into CO_3^{2-} is greatly influenced by increased pH, eventually causing the formed bicarbonate ion to combine with the Ca^{2+} for calcification (Soon, 2013; Anbu *et al.,* 2017; Omoregie *et al.,* 2016). Omoregie *et al.* (2016) reported a research involving the application of MICCP in the treatment of organic soil with a bacterial solution, where it was found that the pH revolved around 9-9.4 while the treatment lasted, suggesting that this range of pH encourages MICCP.

2.6.3 Nutrients

Bacteria involved in bio-calcification require sufficient nutrients, and these are often supplied in the basal medium during fermentation. The nutrients commonly added to the

medium include magnesium in the form of magnesium sulphate ($MgSO_4.6H_2O$), iron in the form ferric oxide (Fe_2O_3), calcium in the form or calcium carbonate ($CaCO_3$), and disodium hydrogen phosphate (Soon *et al.,* 2013).

2.6.4 Dissolved inorganic carbon (DIC)

Dissolved inorganic carbon (DIC) is heavily linked to biocalcification or MICCP. DIC in aqueous solution has four (4) categories namely carbon (iv) oxide (CO_2), carbonic acid (H_2CO_3), ions of bicarbonate (HCO_3^-), and carbonate ions (CO_3^{2-}).

$$CO_2 \longleftrightarrow H_2CO_3 \longleftrightarrow HCO_3^- \longleftrightarrow CO_3^{2-}$$
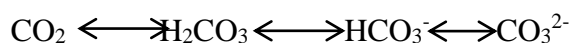
The inter-conversion process that occurs between CO_2 and H_2CO_3 is a slow one, with an equilibration of about 90% in 20 seconds under certain conditions, and the rest of the carbonate cycle instantly equilibrating. Carbonic anhydrase (CA) is renowned to be effective in catalyzing the reversible hydration of CO_2, and while in this aqueous state the CA takes up both the CO_2 and H_2CO_3. Eventually hydrating CO_2 to produce bicarbonate, CA is essential in the DIC uptake and exchange in MICCP (Li *et al*., 2018).

2.7 Carbon (IV) Oxide (CO_2) Sequestration

One of the most abundant greenhouse gases is carbon (iv) oxide (CO_2), a major global warming gas which find its way to the atmosphere as a result of human activity, and a high level of this gas in the atmosphere is largely implicated to have significant contribution to climate change. Although CO_2 when compared to other greenhouse gases, like methane, its strength as a greenhouse gas has been traced to its long term accumulation in the atmosphere largely resulting from the indiscriminate utilization of fossil fuels and falling of trees. Technologies currently in use in the global civil and construction sector are of huge concern environmentally and economically. For example, the processes involved in the manufacture of cement from mining of the lime stones to the finished product gulps a range of 2-3% of the demand of global energy, producing

about 0.73-0.99 tons of CO_2 per every ton of cement produced, and accounting for about 8-10% of the anthropogenic and 3.4% of the total CO_2 emission globally respectively (Zhu and Logan, 2014; Aslam *et al.,* 2018; Castro-Alonso *et al.,* 2019).

This however, has led to a huge threat to the global ecosystem, resulting in impactful climate change which has been one of the leading global challenges whose impact have led to drought, increasing sea levels, floods, hunger and even conflicts, with increasing surge in human population. The problem of global warming and climate change pose unquantifiable threat to the productivity and availability of foods globally, as a result of the rising temperatures (with the predicted temperature expected to rise from 1.8^oC-3.6^oC by 2100) and irregular rainfall (Zhu and Logan, 2014; Aslam *et al.,* 2018). With the human population on an alarming increase, there are growing concerns about the dangers of global warming which is caused by the rising levels of atmospheric CO_2 produced by human processes of indiscriminate tree felling, combustion of fossil fuels and some other industrial processes such as cement production. There is an urgent need to find a way towards having a reduced concentration of the atmospheric CO_2. The process of capturing and sequestration of the CO_2 has been proposed and applied to achieve this objective.

Researchers have come up with different proposals aimed at cutting down the concentration of atmospheric CO_2 and have brought forward some suggestions aimed at achieving this which include a proposal for the adoption of renewable energy sources such as solar and wind energy sources. This option has remained difficult to achieve, prompting a shift to the safer approach of atmospheric CO_2 capture and sequestration. Naturally, this technological approach fixes CO_2 and mineralizes it into carbonated forms such as calcite and aragonite over time. Prompting researchers to employ biological means to bio-mimetically sequester CO_2 by the use of the catalytic carbonic anhydrase, an approach that can successfully convert CO_2 into bicarbonates, which have better

safety, stability and eco-friendliness, thus disposing of the CO_2 without causing any harm to the environment (Dhami *et al.,* 2013).

There is need to reduce the atmospheric CO_2 concentration, and nature too has devised means to reducing this gas such as the sequestration of CO_2 by plants, green algae, cyanobacteria, some bacteria species and also the physicochemical atmospheric natural fixation (Ores *et al*., 2012; Srivastava, 2015). Climate change is primarily caused by the greenhouse gases (GHGs) absorbing and emitting thermal radiations. This has attracted the global attention in the last decade with the United Nations leading the fight against climate change. Recently, the Sustainable Development Goals (SDGs) and the 2019 United Nations General Assembly held in New York dedicated a larger portion of emphasis on the fight against climate change (Biniaz, 2019). One approach to effectively combat this global challenge is the application of biological techniques for carbon sequestration, which has proven to be an eco-friendly and a cheaper alternative to the use of chemicals.

The carbonic anhydrase is a renowned enzyme produced by some bacteria that can effectively and efficiently catalyse the reversible reaction of carbon (iv) oxide (CO_2) hydration and dehydration by the process of inter-conversion between CO_2 and bicarbonate, and transferring hydrogen ions (H^+) between the enzyme's active site and the buffer environment. This processes result in the formation of calcite (Komala and Khun, 2014). The capture and sequestration of CO_2 is a vital technology for the significant reduction of atmospheric CO_2, and the biological approach to CO_2 sequestration that occurs as a result of the natural processes in biological systems has been a game changer in this technology. Carbonic anhydrase has emerged as a leading enzyme with a huge ability to capture and sequester CO_2, and it is a good alternative for the reduction of atmospheric CO_2. This is achieved through the process of hydrating CO_2 and then

subsequently producing bicarbonate (Ores *et al*., 2012). The ability to sequestrate atmospheric CO_2 through calcium carbonate formation is one very crucial attributes of some microorganisms. Technologies involving the bio-deposition of calcium carbonate have already been employed for the sand columns consolidation, for remediation of limestone monuments and to a smaller extent recently, and for the prevention and remediation of cracks in concrete (Vahabi *et al.,* 2018). This emerging new technology of capturing and sequestering carbon using biological means such as the utilization of microbial enzymes has huge edge over conventional approaches to carbon sequestration (Panchami *et al.,* 2019).

Carbonic anhydrases' ability to reversibly catalyze the hydration of carbon (iv) oxide and dehydration of the bicarbonate ion is a key quality that qualifies it as a leading candidate in the sequestration of CO_2. The approaches currently employed to trap and sequester CO_2 have relatively high cost implications and often demanding huge energy consumption, and this outweighs end product of CO_2 removal. Thus the ability of CA to hydrate CO_2 is of great importance to finding a more environmentally friendly and less expensive approach for the capture and transformation of CO_2, and current researches are ongoing in finding ways of employing carbonic anhydrases in large scale industrial settings for the purpose of sequestering carbon. This ultimately will have positive impact on global warming and provide for a sustainable environment (Gonzalez and Fisher, 2014).

Srivastava (2015) reported that bacteria utilize CO_2 for growth in addition to producing carbonated end products in the form of calcite. As reported by Srivastava (2015), certain strains of bacteria such as *Bacillus cereus*, *B. subtilis*, *B. pasteurii*, *Pseudomonas aeroginosa*, *P. fluorescens* and *P. putida* have the ability to precipitate carbonates, mostly

when presented with different factors such as salt concentration, alkaline pH and the adequate medium composition.

The process of converting CO_2 into calcium carbonate is an environmentally friendly approach that involves the sequestration of CO_2. A key enzyme in this biochemical process of CO_2 sequestration and calcite formation is the carbonic anhydrase (CA), a metalloenzyme containing zinc that catalyzes the reversible reaction of CO_2 hydration. (Kanbar, 2008; Dhami *et al*., 2013). Ibrahim *et al.* (2016) also reported that CA enzymes are zinc metalloenzymes whose primary function is the catalysis of the reversible hydration of CO_2 leading to the formation of bicarbonate and proton, this reversible reaction the authors added in the absence of an enzyme catalyst, is relatively slow. In the same vein, Bond *et al*. (2001) and Kanbar, (2008) reported that the major transformation for CO_2 sequestration for calcification include the dissolution in an aqueous phase of the CO_2 followed by hydration which is enhanced by the CA, ionization and then in the presence of a calcium source for the formation of calcium carbonate.

2.8 *Alcaligenes faecalis* subsp. *parafaecalis* strain G

Since its establishment in the early 20th century to cater for aerobic, Gram negative, coccobacillary rods that are motile within the family *Alcaligenaceae*, the *Alcaligenes* genus have gone through a lot of changes, it is presently narrowed down to the species *Alcaligenes faecalis* and subdivided into *A*. *faecalis* subsp. *faecalis*, *A*. *faecalis* subsp. *parafaecalis*, *A*. *defragans* and *A*. *latus* (Van Trappen *et al.*, 2005; Lu *et al*., 2017). *Alcaligenes* specie strains are found in water, soil and the general environment including close association with humans. Most bacteria of this genus are Gram negative and rod shaped, and they exhibit diverse properties and applications but not much has been explored for it CA producing ability and biocalcification. This research study identifies the strain as a potential beneficial bacterium in biocalcification of concrete. They are

generally regarded to as safe (GRAS) as they are usually not pathogenic but can sometimes be opportunistically pathogenic (Rehfus and Urban, 2005; Kumar *et al*., 2013). They have been reported to demonstrate diverse ability to bioremediate pollutants such as the conversion of arsenic to a less toxic form of arsenate. In addition, they have also been reported to have high heavy metals tolerance ability, applicable in the production of nanoparticle detergents, bioplastics, and also a very good candidate in biocontrol applications (Basharat *et al*, 2018).

This perhaps explains the ability of *Alcaligenes faecalis* subsp. *parafaecalis* strain G to be able to hydrolyse paranitrophenylacetate (pNPA). Biotechnologically, the genus *Alcaligenes* is significant. With some strains reported to have the ability to degrade phenol while some are capable of producing poly-β-hydroxybutyrate. In addition, their ability to tolerate heavy metals makes them a leading organism to be exploited for bioremediation (Lu *et al*., 2017).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Samples Collection

Soil samples used in this research study were collected with the aid of a hand trowel, from the surface soil of two (2) selected concrete construction sites, Garima block construction site and Kotangora plaza construction site, both in Bosso Local Government Area in Minna, Niger State. The choice of this study site was informed by the fact that construction works were already going on in the sites, and that the microorganism(s) of interest could be easily isolated from the sites since they are already acclimatized to utilizing the conditions. The sample sites are located between $9^{o}38'47.5"N$ $6^{o}32'39.7"E$. The samples of soil were collected inside sterile sample collection bottles following the methods described by Li *et al.* (2004) and, they were transported to the Department of Microbiology Laboratory, Federal University of Technology, Minna.

3.2 Media Preparation

The media preparation was carried out first by preparing the broth-peptone agar for the isolation of the bacteria and the preparation of para-nitrophenyl acetate (pNPA) which was used as the substrate for the carbonic anhydrase bacteria isolation.

3.2.1 Preparation of broth-peptone agar

Broth-peptone agar used for the isolation of the potential carbonic anhydrase producing soil bacteria was prepared following the method described in Li *et al.* (2004) with slight modification. The media was prepared in sterile Petri dishes by measuring in 100 mL of distilled water; peptone (1.5 g), agar-agar (1.5 g), nutrient agar (2.8 g) and $CaCO_3$ (0.6 g) in a 500 mL conical flask. The resulting mixture was shaken to allow thorough dissolution, and autoclaved at 121°C for 15 minutes, and then left to cool down and poured aseptically into the sterile Petri dishes and swirled for even distribution on the plates.

3.2.2 Preparation of the para-nitrophenyl acetate (pNPA)

The para-nitrophenyl acetate (pNPA) otherwise referred to as 4-nitrophenyl acetate is a form of indicator salt employed to determine the carbonic anhydrase producing ability of bacterial isolates by the hydrolyzing the pNPA into paranitrophenol (pNP). It is also used to test for the CA activity.

For the purpose of this study, using the method of Ibrahim *et al.* (2016) with slight modification, 25 mg of the pNPA salt was dissolved in 2 mL of acetone, and this was continued until about 1.8 g of the salt was completely dissolved. A 3 mM concentration of the salt was prepared by adding sterile distilled water, and stored aseptically in a sterile bottle for further application and use.

3.3 Isolation of Bacteria

3.3.1 Isolation and screening of bacteria

The bacteria were isolated using the methods described by Aggarwal *et al*. (2015) and Anyadoh *et al*. (2017) with slight modifications; the soil samples were serially diluted in 10-fold by adding one gram (1 g) each of the two soil samples into well-labeled test tubes with nine milliliters (9 mL) of sterile distilled water respectively. This was followed by a thorough shaking of the resulting mixture in order to homogenize the mixture. The dilution was continued by transferring one milliliter (1 mL) of the supernatant from the first dilution tube of each sample into 9 mL of sterile water respectively, and it was carried on in that order for both samples up till the sixth dilution factor (10^{-6}).

Following the protocols of Li *et al*. (2004) with slight modification, 1 mL of the third dilution factor of each soil sample was inoculated by pour plating in the broth-peptone agar plate; 1 mL of dilution three of each soil sample was added into the sterile Petri dishes respectively and the molten sterile broth-peptone agar medium prepared was added and the plates were swirled gently to allow for an equal distribution. The broth-peptone agar plates were then left to solidify and then were sprayed with the prepared 3 mM p-NPA. The plates were then left for 15 minutes to allow for a complete incorporation of p-NPA, and then incubated by placing in the incubator for a period of 28-48 hours in an inverted position.

Colonies of bacteria with the CA producing ability presents with a dense yellow colouration as a result of the hydrolysis of p-NPA into para-nitrophenol (pNP). This method employed by Li *et al.* (2004) seeks to serve two purposes; first as a means of isolation of the bacteria and, secondly to screen for the potential of the bacteria isolates to produce carbonic anhydrase.

Upon the completion of the incubation period, two colonies of bacteria that were of interest were picked and carefully streaked on sterile broth-peptone agar medium plates

to obtain pure isolate, and upon inoculation, the plates were again sprayed with the p-NPA solution and allowed to stand for 15 minutes so as to ensure the complete infusion of the p-NPA. The plates were incubated for 24- 48 hours at a temperature of 37°C. The isolates obtained were then stored in nutrient agar slants for further characterization and confirmation (Li *et al.,* 2004).

3.3.2 Confirmatory test for carbonic anhydrase production

Screening for carbonic anhydrase was further carried out to authenticate the initial screening during bacterial isolation, by following the slightly modified method of Alshalif *et al.* (2018). The bacterial isolates were aseptically inoculated into a freshly prepared broth-culture medium composed of nutrient broth and 3 mM p-NPA. A broth-culture medium without inoculation was also set up to serve as the control. The test tubes were left for two (2) weeks in an incubator with shaker at 37°C. Observations were made for colour change and findings were recorded.

3.3.3 Characterisation and identification of bacterial isolates

Gram staining and some other biochemical tests (such as oxidase, citrate utilization, catalase, indole, motility, methyl red and Voges-Proskauer, and carbohydrate utilization tests, were conducted after which anaerobic culturing of isolates was carried out to determine the characteristics of the isolates using the slightly modified methods described in Cheesbrough (2006) and Aggarwal *et al*. (2015). Identification of the isolates was then completed by comparing their morphological and biochemical characteristics with that of known taxa.

3.3.3.1 Gram staining

This is a form of differential staining procedure employed to characterize, identify and classify bacterial cells by their reaction to the Gram's reagents and their morphology. The Gram staining technique involves the application of two stains (the primary and

secondary stain), and the bacterial cells differentiation depends on which of the stains are retained by the cells, based on the structural composition of their cell wall.

In this study, pure isolates were stained using the Gram's reagents under sterile conditions; the wire loop was flamed until red hot, then cooled, and used to pick from the stored bacteria isolates. A drop of normal saline was placed on a clean, grease-free glass slide and the picked isolate was smeared onto the normal saline. The smear was air-dried and then heat-fixed, and flooded with crystal violet and left for 60 seconds. The crystal violet was drained off and water was run over the stained smears.

Furthermore, Lugol's iodine solution was used to flood the stained smears, and left for 60 seconds, and then tap water was gently run on it for proper rinsing. The stained smears were decolourized by adding a few drops of 95% ethanol and left for less than 30 seconds, and then rinsed off again. The decolorized smears were counter-stained by flooding the smears with safranin, and left to stand for 30 seconds, and then gently rinsed off using distilled water, after which it was air-dried. The slides were afterwards viewed under light microscope using oil immersion objective lens ($\times$100), bacteria cells were observed and the observations recorded (Cheesbrough, 2006)

3.3.3.2 Catalase test

This test is employed to identify microorganisms capable of breaking down hydrogen peroxide (H_2O_2), a toxic form of oxygen by the use of the enzyme called catalase. These organisms are able to survive in an environment containing H_2O_2, by producing catalase which aids the neutralization of H_2O_2 by catalysing its breakdown to water and oxygen. The test was conducted by introducing the test organism into a 3% H_2O_2; bubbles formation is an indication of catalase production by the organism, while a lack of bubble formation indicates otherwise.

In this study, two (2) drops of 3% H_2O_2 was aseptically placed on 3 well-labeled clean and grease free slides. The bacterial isolates to be tested were with the aid of a clean, sterile glass rod and smeared on the labeled slide containing the H_2O_2, except the slide labeled control. Observations were made on the slides and recorded (Cheesbrough, 2006).

3.3.3.3 Indole test

This is a biochemical test employed to distinguish between closely related bacteria species. It aids in the identification of bacteria species capable of hydrolysing the amino acid tryptophan *via* the expression of the enzyme trytophanase, releasing the indole ring of the amino acid. The organisms capable of expressing this trytophanase are classified as positive to indole (characterized by the formation of a pink to red ring on the surface of the inoculated medium (tryptophan broth) after the addition of Kovac's reagent), while the ones that are not capable are said to be negative to indole.

In this study, the bacteria isolates for test were aseptically inoculated into 5ml of sterile peptone broth and aerobically incubated for 48 hours at 37°C. The Kovac's reagent was prepared by the dissolution of 5 g of dimethyl amino-benzaldehyde in 75 mL of amyl-alcohol and 25 mL of concentrated hydrochloric acid (HCl). After the incubation period, the Kovac's reagent was added in a drop-wise manner to each cultured broth and shaken gently. Observations were made on the cultures and the findings recorded (Cheesbrough, 2006).

3.3.3.4 Oxidase test

This test is employed to identify and differentiate microorganism containing a protein called cytochrome c, which is functionally involved in the electron transport chain of the mitochondria during aerobic respiration, through the production of an intracellular enzyme called cytochrome c oxidase. In this study, 3 drops of the oxidase reagent was aseptically placed on a sterile filter paper, the test bacteria isolates were smeared on the

wet filter paper using sterile glass rod. Observations were made and the findings were recorded (Cheesbrough, 2006).

3.3.3.5 Citrate test

The test is used to determine the ability of bacteria carry out citrate utilization as a sole carbon source. It involves microbial culturing in a Simmon citrate agar; a medium containing citrate as the only source of carbon and an inorganic ammonium salt as its nitrogen source. In this study, the test bacteria isolates were aseptically inoculated using a sterile wire loop by streaking on a sterile Simmon citrate agar slant, and on one other slant which served as a control. The inoculated agar slants were aerobically incubated for four (4) days at 37°C, after which the slants were observed, and compared to the control slant, and the findings recorded (Cheesbrough, 2006).

3.3.3.6 Motility test

This test is employed to determine an organism's motility ability. In this study, a sodium chloride-peptone agar was prepared by prepared by adding 10 g of peptone, 5 g of agar agar, 5 g of sodium chloride (NaCl)/1000 mL of distilled water and sterilized by autoclaving at a temperature of 121°C for 15 minutes. A sterile straight wire loop was used to aseptically pick the bacteria isolates and inoculated on the sodium chloride-peptone agar slants by stabbing with a sterile needle just right at the middle of the medium to a depth of about 2 cm, except on the control slant. The inoculated agar slants were then aerobically incubated at 37°C, for 18-24 hours. Observations were made on the slants and the findings were recorded (Cheesbrough, 2006).

3.3.3.7 Carbohydrate utilization test (production of acid and gas from carbohydrate)

This test is carried out to separately test for the ability of a bacterium to aid the breakdown of sugars like glucose, sucrose, mannose, maltose, lactose, and fructose, and also their pattern of fermentation so as to differentiate it from other species. Gas and/or acid

production is an indication of a fermentation reaction; acid production is indicated by a colour change (from red to yellow) of the phenol red (serving as the indicator) in the test medium, while gas production is indicated by the gaseous accumulation in the Durham tube used.

In this study, bacteria isolates were inoculated aseptically into the test tubes containing sterile phenol red-peptone-sugar broth (phenol red peptone broth containing sucrose, fructose, lactose, D-glucose, D-mannitol, arabinose, sorbitol, D-mannose) and Durham tubes, except the tubes that served as control for all the different peptone-sugar broth. The inoculated broths were then aerobically incubated at 37°C for a period 24-48 hours. After incubation, observations were made of broth and Durham tubes and the findings recorded (Cheesbrough, 2006).

3.3.3.8 Methyl red and Voges-Proskauer test

This test is carried out for the identification of bacteria species based on their ability to produce sufficient acid during glucose fermentation. This reaction involves the conversion of glucose into pyruvate and a further metabolic reaction through the mixed acid pathway for the production of a stable acid (which is dependent on the microbial species involved), lowering the pH of the test medium (methyl red-Voges-Proskauer broth), and changing the colour from yellow to red upon the addition of methyl red. The Voges-Proskauer test is employed for the determination of an organism's ability to produce acetoin during the fermentation of glucose. This is carried out by the addition of alpha-naphthol reagent which functions to convert the acetoin into diacetyl when in the presence of atmospheric pressure and then condenses with quinidine, giving the test broth a pinkish red colouration.

In this study, the isolates were aseptically inoculated into 2 test tubes containing 2ml of sterile glucose-phosphate-peptone broth, and incubated 37°C. Four (4) drops of methyl

red was then added to the cultured broth using Pasteur's pipette, and was gently shaken to allow proper mixing. Observations were then made and the findings recorded. In addition, in the 2 other cultured VP broth, 1 mL of 40% potassium hydroxide (KOH) solution and 3 mL of 5% alcoholic alpha-naphthol was aseptically added and was shaken properly, it was left to stand for 4 minutes. Observations were made and findings were recorded (Cheesbrough, 2006).

3.3.3.9 Anaerobic incubation

This test is used to determine the ability of the test bacteria to grow and metabolise when in an environment that lacks oxygen. Bacteria with such ability present visible colonies on the agar plates, while those lacking such ability do not form visible colonies on agar plate. In this study, bacteria isolates were streaked on sterile nutrient agar plates using a sterile wire loop and placed in an anaerobic jar. The plates were anaerobically incubated for 18- 24 hours, after which observations were made on the plates and the observations recorded (Cheesbrough, 2006).

3.3.4 Molecular identification of the bacterial isolate GA (B)

DNA extraction, polymerase chain reaction (PCR) amplification process, extracted DNA integrity test, and the methods for purification and sequencing of the extracted DNA were carried out according to the method described by Frank *et al*. (2008) for the molecular identification of isolate GA (B).

3.3.4.1 DNA extraction

Using the protocol described in Frank *et al*. (2008), DNA was extracted. Briefly, single colonies of the bacterial sample grown on medium were transferred to 1.5 mL of liquid medium and cultures were grown on a shaker at 28ºC for 48 hours. This was followed by the centrifugation of the cultures for five (5) minutes 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 micro Liters (15 μL) of 20% SDS and 3 μL of Proteinase K (20 mg/mL) were then added, and the mixture was incubated at 37ºC for 1 hour, 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 65ºC for 10 minutes and placed on ice for 15 minutes. This was followed by the addition of an equal volume of chloroform: isoamyl alcohol (24:1) which was then incubated on ice for 5 minutes and centrifuged for 20 minutes at 7200 g. The aqueous phase was then moved to a new tube, adding isopropanol in a ratio of 1:0.6 and precipitating the DNA for 16 hours at -20ºC. Collection of the DNA was carried out by centrifugation for 10 minutes at 13000 g, washed with 500 μL of 70% ethanol, air-dried for around 3 hours at room temperature and finally dissolved in 50 μL of TE buffer (Frank *et al.,* 2008).

3.3.4.2 Polymerase chain reaction (PCR)

The cocktail of preparation for PCR sequencing comprised of 10 μL of 5x GoTaq colourless reaction, 3 μL of 25 mM $MgCl_2$, 1 μL of 10 mM of dNTPs mixture, 1 μL of 10 pmol each 27F 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 minutes at 94°C, followed by a 30 cycles made up of 94°C for 30 seconds, 50°C for 60 seconds and 72°C for 90 seconds; and a final termination for 10 minutes at 72°C, and chill at 4ºC GEL (Frank *et al.,* 2008).

3.3.4.3 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 minutes 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 transfered into the tray. To form the wells, the gel was allowed to stand for 20 minutes in order to solidify. The 1XTAE buffer was poured into the gel tank to barely cover the gel. Two micro Liter (2 µL) of 10X 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 minutes 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).

3.3.4.4 Purification of amplified product

After the gel integrity, 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 each about 40 µl PCR amplified product in a new sterile 1.5 µL tube of eppendorf, mixed thoroughly by vortexing and kept at -20°C for at least 30 minutes. Centrifugation for 10 minutes at 13000 g and 4°C was followed by removal of the supernatant (invert tube on trash once) after which the pellet were washed by adding 150 µL of 70% ethanol and mixed, then centrifuged for 15 minutes 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 for 10-15 minutes. It was then re-suspended with 20 µL of sterile distilled water and kept in -20ºC prior to sequencing.

The purified fragment was checked on a 1.5% Agarose gel ran on a voltage of 110 V for about 1 hour as previous, to confirm the presence of the purified product and quantified using a nanodrop of model 2000 (Frank *et al.,* 2008).

3.3.4.5 Sequencing

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.

3.4 Large Scale Preparation of Crude Carbonic Anhydrase

For a large scale production of the crude carbonic anhydrase from the isolated bacterial isolates, an inoculum was prepared of the isolates and then a fermentation medium was formulated, which served as a composition of substrates to enhance the enzyme production.

3.4.1 Inoculum preparation

Applying the method described in Li *et al.* (2004) which was modified slightly, a transition was made of the bacterial isolates from a solid agar medium to a broth medium. A nutrient broth medium which served as the basal medium was prepared in a 250 mL conical flask by measuring according to the manufacturer's instruction in a 100 mL distilled water, and autoclaved for 15 minutes at 121°C and allowed to cool. A sterilized wire loop was used to aseptically pick the bacteria isolates from the slants and inoculated into the broth. The conical flask was then corked, swirled for proper mixing and incubated for 4days in a rotary shaker at 150 rpm at a temperature of 30°C and observed for growth.

3.4.2 Basic culture medium

The basic culture medium otherwise called the fermentation medium was formulated according to the method described in Zhang *et al.* (2011) which was modified slightly for

the purpose of this research. It was composed of; sucrose 5.0 g/L, disodium hydrogen phosphate, Na_2HPO_4 2.0 g/L, magnesium sulphate, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, calcium carbonate, $CaCO_3$ 0.1 g/L, Iron (III) chloride, $FeCl_3 \cdot 6 H_2O$ 0.005 g/L and Cement 5 g/L. These were measured in a 1000 mL conical flask and 500 mL of distilled water was added and autoclaved for 15 minutes at 121°C, it was cooled and after which 20% (v/v) inoculums from the already prepared inoculum was aseptically added with the aid of sterile pipette. The culture was incubated for 7 days at 32°C in a rotary shaker at 150 rpm.

After the completion of incubation period, the final liquid culture was centrifuged for 15minutes at 7000 rpm in order to remove the bacterial cells. The resulting supernatant was separated and then filtered using a 0.22 μm pore size filter membrane in order to obtain a cell free supernatant. The cell free supernatant was taken as the crude carbonic anhydrase enzyme produced, and stored in the refrigerator at freezing temperature for further determination of enzymatic activity, after which the production was continued until about 4 L of the crude CA were produced.

3.5 Determination of Carbonic Anhydrase Activity

The enzyme activity was carried out by following and modifying the methods used by Muller *et al.* (2013). A reaction mixture made up of phosphate buffer (with a concentration of 0.1 M at pH 7.2) and 3 mM concentration para-nitro phenyl acetate (pNPA). 0.1 mL of the crude CA was added to 0.9 mL of the prepared reaction mixture in a test tube. A blank preparation was made without the substrate to serve as a control or blank. Observation was made for colour change and the measurement taken using the ultra violet spectrophotometer (UV spec) at a wavelength of 412 nm for 5 minutes. Para-nitrophenol curve (pNP curve) was prepared by dissolving in concentrations of 1 mg, 2 mg, 3 mg, 4 mg and 5 mg of paranitrophenol in 1 mL of distilled water in test tubes, and shaken for proper dissolution. Reading was also taken on the UV spectrophotometer at a wavelength

of 412 nm and the values recorded. The generated results were used to produce a standard curve, and the pNP standard curve was used for the calculation of the CA activity. The enzyme activity was given in mmoles/mL^{-1}/secs^{-1}

3.5.1 Determination of optimum conditions for CA activity

Optimization was performed in order to ascertain and understand the effects of some varying conditions that enhance the activity of carbonic anhydrase produced by the bacterial isolates. The parameters tested for optimization include the substrate, temperature and pH.

3.5.1.1 Determination of optimum substrate concentration for CA activity

Para-nitrophenylacetate (pNPA) was used to test for the optimum concentration of substrate for the optimum CA activity. The CA activity was determined by incubating the enzyme at seven (7) different concentration of the substrate from 1 mM to 7 mM for 5 minutes, and measuring the absorbance increase at 412 nm. The reaction was conducted in triplicate following the protocols described in Capasso *et al.,* (2012) with slight modification.

3.5.1.2 Determination of optimum temperature for CA activity

To ascertain the temperature optimum for carbonic anhydrase activity, the pNPA was used as substrate and the enzyme was incubated at varying temperature ranging from 30 to 80ºC for 5 minutes and measuring the increase in absorbance at 412 nm. The reaction was carried out in triplicate as described in Capasso *et al.* (2012) and Panchami *et al.* (2019) with slight modification.

3.5.1.3 Determination of optimum pH for CA activity

The effect of pH on the activity of carbonic anhydrase produced was determined as described in Zhang *et al.* (2011), Capasso *et al.* (2012) and Panchami *et al.* (2019), with slight modification. The crude enzyme was incubated at varying pH ranging from 5.5, 7.5,

8.5 and 9.5 for 5 minutes, and increase in absorbance was measured at 412 nm to determine the optimum pH for CA activity.

3.6 Concrete Preparation

The concrete was prepared in the right mix of components according to the recommended specification BS1881 (1993), BS EN 12390-3/5 (1990).

3.6.1 Materials for bio-concrete production

As described by Patil *et al.* (2016) the materials included cement, fine aggregates (sand), coarse aggregate (stones or pebbles), and water, and the crude carbonic anhydrase.

The cement used was Ordinary Portland Cement that was Standards Organization of Nigeria (SON) certified and commercially available. It was purchased from the market in Bosso Local Government Area, and tested in the Civil Engineering laboratory of the Federal University of Technology, Minna to determine the specific gravity, as described in Satinder *et al.* (2017).

The sand was sourced from a natural river sand whose fineness conformed to building standards and the specific gravity was also tested in the laboratory, the coarse aggregate source was of 20mm sizes with fine modules whose specific gravity was also tested and conforming to building standards BS 1377-2 (1990), and the water was from approved water source that is relatively pure as described in Patil *et al.* (2016).

3.6.2 Specific gravity test

This test was carried out on the soil sample in conformity with BS 1377 (1990). The materials used to carry out the specific gravity tests include a dish, an electronic weighing balance, a scoop, a measuring cylinder, a shallow tray, and a drying cloth.

In this study, three (3) clean density bottles were weighed and the weights recorded, after which clean water was used to fill the density bottles, their weights taken and then recorded.

Little soil sample was poured inside the density bottles; clean water was used to fill the bottles, thoroughly shaken, and filled up to the calibrated brim with water. The bottles were then allowed to stand for 10 minutes so as to let out the air that was trapped inside and the values were read and recorded. The specific gravity was calculated using the expression in equation 3.1.

$$Gs = \frac{W1-W2}{(W2-W1)(W3-W4)}$$

(3.1)

Where;

Gs: Specific gravity

W_1: Weight of the density bottle without any content

W_2: Weight of density bottle containing dry soil

W_3: Weight of density bottle containing soil and water

W_4: Weight of density bottle containing water only.

3.6.3 Sieve analysis of soil sample

The sieve analysis of the soil sample was carried out in accordance with BS, 1377 (1990). The materials used in conducting the sieve analysis include oven, sieve shaker, an electronic weighing balance, a rifle, a weighing pan, a standard sieve set of varying sieve sizes, sieve cover and test samples of fine aggregate, coarse aggregate (10 mm sizes) were used.

In this study, 300 g of the soil sample was weighed out and placed in labeled can, weighed and left to soak for 24 hours. Washing of the soil sample was carried out thoroughly until clean water passes through using a sieve with the size of 75 μm. The retained soil sample was removed carefully on the pan and was then transferred to another pan for oven drying.

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The weighing balance was then set up and initialized. The empty sieves set was each weighed and the values recorded correspondingly. The sieves were then arranged in an order of descending sizes, beginning from the largest to the smallest sieve to form the set of sieves as shown in plate I. The sample already oven dried was then transferred into the sieves set placed on the mechanical shaker. The shaker was turned on for a period of 10 minutes, each of the sieves with the retained sample was measured on the electronic weighing balance and the readings were taken and recorded correspondingly. The percentage retained or passes were calculated using equations 3.2 and 3.3 respectively.

$$\text{Percentage Retained} = \frac{(Weight\ of\ soil\ sample\ on\ each\ sieve\ )}{\text{Total weight of soil sample}} X100 \tag{3.2}$$

$$\text{Percentage passing} = 100 - \text{Cummulative weight retained} \tag{3.3}$$

Plate I: Set of sieves for particle size distribution

3.7 Mix Design for the Concrete

This is a process of selecting the right materials for concrete and then determining their relative quantities with the objective of producing, as economically possible, concrete of certain minimum characteristics such as strength, durability and a required consistency (Neville, 2003). The concrete mix design was determined using the Nominal Concrete mix ratio of 1:2:4 for cement:sand:aggregate.

For a reduced water cement ratio of 0.50:

$$\frac{w}{c} = 0.50$$

Where,

w is water content; c is cement content

Cube size $= (0.15 \times 0.15 \times 0.15)m^3$

$$= 0.000375m^3$$

(3.4)

Allowance for waste $= 5\%$

Data for Design:

Density of cement, $\rho_c = 3.15$

Density of water, $\rho_w = 1000$

Density of fine aggregate, $\rho_{fa} = 2.6$

Density of coarse aggregate $\rho_{ca} = 3.15$

Bulk Density of cement $\gamma_c = 1440$

Bulk Density of fine aggregate $\gamma_{fa} = 1500$

Bulk Density of coarse aggregate $\gamma_{ca} = 1700$

Using the Mix ratio of 1:2:4

$$\frac{1440}{1440} : \frac{2 \times 1500}{1440} : \frac{4 \times 1700}{1440}$$

$$1 : 2.08 : 4.72$$

$$1m^3 = \frac{0.5c}{1000} + \frac{1.0c}{3150} + \frac{2.08c}{2600} + \frac{4.72c}{2700}$$

$$= \frac{1.575c + 1.0c + 2.52c + 5.507c}{3150}$$

$$= \frac{10.602c}{3150}$$

$$c = \frac{3150}{10.602}$$

$$= 297.11 \text{ kg}$$

$1m^3 \ of \ cement = 297.11$ kg

Add 5 % for waste, i.e. 1.05x297.11 = 3.11.97 kg

Therefore,

$1m^3 \ of \ cement = 311.97$ kg

Volume of 1 cube $= 0.0003375 m^3$

$$0.0003375 m^3 = \frac{0.0003375 \times 311.97}{1 m^3}$$

$$= 1.06 \text{ kg}$$

Therefore,

1 Cube of cement $= 1.06$ kg

1 Cube of fine aggregate $= 1.06$ kg$\times 2.08 = 2.21$kg

1 Cube of coarse aggregate $= 1.06$ kg$\times 4.72 = 5.01$kg

1 Cube of coarse aggregate $= 1.06$ kg$\times 0.5 = 0.53$kg

Table 3.1: Mix design of concrete

25 Cubes: 12 Cubes for (100% Water 0% Crude CA) + 13 Cubes (20% Water 80% Crude CA)

S/No	Materials	Weight (kg)	S/No	Materials	Weight
1	Cement	$1.06 \times 12 = 12.72$ kg	1	Cement	$1.06 \times 13 = 13.78$ kg
2	Fine Aggregate	$2.21 \times 12 = 26.52$ kg	2	Fine Aggregate	$2.21 \times 13 = 28.73$ kg
3	Coarse Aggregate	$5.01 \times 12 = 60.12$ kg	3	Coarse Aggregate	$5.01 \times 13 = 65.13$ kg
4	Water	$0.53 \times 12 = 6.36$ kg	4	Water	$0.20 \times 0.53 \times 13 = 1.378$ kg
5	Crude CA	0 kg	5	Crude CA	$0.80 \times 0.53 \times 13 = 5.512$ kg

Summary

A) 12 Cubes: 100% Water 0% Crude CA)

Cement = 12.72 kg

Fine Aggregate = 26.52 kg

Coarse Aggregate = 60.12 kg

Water = 6.36 kg

Extract= 0 kg (No Crude CA)

B) 13 Cubes: 20% Water 80% Crude CA)

Cement = 13.78 kg

Fine Aggregate = 28.73 kg

Coarse Aggregate = 65.13 kg

Water = 1.378 kg

Crude CA = 5.512 kg

3.7.1 Concrete mixing procedure

For the purpose of this study, mechanical concrete mixing technique was used in mixing the concrete. It is the process of mixing ingredients of concrete with a motorized concrete mixer, and it has a very high effectiveness for fulfilling the demands in short mixing time, with optimum consistency and producing a concrete with homogeneous quality. Apparatus used for the mixing include: electric weighing balance, head pan, shovel, mixing tray, and bucket, and hand trowel, scoop and concrete mixer machine. The quantities of the various concrete materials (cement, fine and coarse aggregate, crude enzyme, water) were measured as calculated in the mix design. The inner surfaces of the concrete mixer were

wet, and the coarse aggregates were placed in the mixer then followed by sand then cement. The materials were mixed in dry state in the mixing machine for some minutes. After proper mixing of dry materials, specific water and crude enzyme extract was added while the machine was in motion. The mixture was then mixed for some more minutes.

3.7.2 Concrete cubes casting

For the purpose of this study, the concrete iron moulds of 150 mm × 150 mm × 150 mm internal dimensions were used, according to BS 1881 (1993). The moulds were first polished with oil lubricant to cause a reduction in the friction and also aid the demoulding of the cubes from the iron moulds easily. Once ready, they were filled with the prepared concrete mix in three (3) layers and each layer tampered 25 times. Proper finishing of the top surface of the concrete cubes was done with the aid of a hand trowel and labeled after a while for proper identification. The concrete cubes were left to set for 24 hours after which they were demoulded as presented in Plate II.

Plate II: Moulded concrete cubes

3.7.3 Curing of concrete cubes

The concrete cubes after demoulding were put in a clean curing tank and filled with clean water to allow them go through the process of curing for specified ages of 7, 14 and 28 days. The concrete cubes during curing are presented in the Plate III below.

Plate III: Curing of demoulded concrete cubes

3.8 Concrete Tests

The following test was conducted on the concrete cubes including, compressive strength test according to BS EN 12390-3/5 (2009) and the Scanning Electron Microscopy (SEM).

3.8.1 Compressive strength testing

The compressive strength is used to determine the strength and hardness of concrete (Gandhimathi *et al.,* 2015; Satinder *et al.,* 2017). The Compressive strength of the concrete cubes was carried by crushing the concrete cubes with respect to the age of interest The materials used for the crushing process include crushing machine, weighing balance (all available in the Civil Engineering Department, Federal University of Technology, Minna), and the concrete cubes specimens.

After the concrete cubes were cured to the specified ages in days 7, 14 and 21, they were removed from the curing tank for crushing, and air-dried, weighed and axially placed in the crushing machine with the two sides of the cube faces in contact with the platen of the

crushing machine as specified in BS 1881 part 116:1983. The machine was connected to the power source and turned on, and carefully operated to crush all the concrete cubes, the point of failure of the concrete cubes upon load application (the final crushing strength indicated by the movement stoppage of the reading gauge) was noted and recorded as the crushing load (KN). The compressive or crushing strength for 7, 14, and 28 days respectively were determined in conformation with IS 516:1959 and calculated from the expression in the equation 3.5 presented below:

$$\text{Compressive strength} = \frac{Load\ of\ failure}{cross\ sectional\ area\ of\ the\ cube} \hspace{3cm} (3.5)$$

The percentage (%) strength gained was calculated as:

<u>Compressive strength at day 28 - compressive strength at day 7</u>   X 100

Compressive strength at day 28.   (3.6).

The Plate IV below shows the concrete cube in the crushing machine ready to undergo the compressive strength testing.

Plate IV: Compressive strength testing machine with concrete cube set for crushing

3.8.2 Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) is a test procedure that is used to scan a test sample with beam of electrons over a surface, producing an image in a magnified form for analysis and interpretation. This beam of electrons forms an interaction with the test sample to produce different signals which can be employed to get information about the sample's surface topography, composition, micro-, and failure analysis of solid inorganic material samples.

In this study, SEM (EVO LS 10, CARL ZEISS, Germany) was used to measure the surface morphology and composition of calcite precipitates on the concrete specimen after the 28th day curing period, and this was used to determine the compactness and carbonation of the prepared concrete.

For the purpose of this study, concrete samples for SEM were collected from the broken pieces of cubes obtained from compressive strength test. Following the method described by Golding *et al.* (2016), the concrete samples were first dried using hexamethyldisilazane (HMDS) and at a temperature of $50^{o}C$ in an oven to remove any trace of moisture for about 6-8 hours 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 aluminium subs, and then placed in the stage or sample holder. The samples were imaged under a high vacumm at a current of 10.00 kv, 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 minutes and 40 seconds, and the images were 2560 x 1920 pixels each. Lastly, the recorded SEM images ranged from 3,000 x to 20,000 x, and the results recorded for interpretation. An Energy Dispersive Spectrometer (EDS) connected with SEM was used to detect the components of precipitation.

3.9 Data Analysis

For the purpose of this study, the data results obtained from this study were analyzed using one way analysis of variance (ANOVA) followed by SPSS version 23. The P values <0.05 were considered statistically significant while P values >0.05 were statistically insignificant.

CHAPTER FOUR
4.0   RESULTS AND DISCUSSIONS

4.1 Results

4.1.1 Carbonic anhydrase (CA) producing potential of bacterial isolates

Two bacterial isolates were obtained from the soil sample from Garima construction site, and were further screened for their carbonic anhydrase (CA) producing ability. The pictures of the isolates are presented in Plates V and VI.

yellow colonies showing the hydration of pNPA broth-peptone agar

Plate V: Isolate GA (A) obtained from the soil sample showing reaction to pNPA

Orang/peach colonies showing reaction to pNPA

Broth-peptone agar Petri plate

Plate VI: Bacterial Isolate GA (B) obtained from the soil sample showing reaction to pNPA

4.1.1.1 Hydrolysis of para-nitrophenyl acetate (pNPA) by the bacterial isolates

The two bacterial isolates screened for CA producing ability showed positive results to the hydrolysis of the substrate pNPA. The isolates were able to hydrolyse the pNPA to para-nitrophenol (pNP) and acetate. This caused a density in the otherwise yellow colouration, while the control remained unchanged after two weeks. The pictorial representations of the confirmatory test for CA production by the bacterial isolates are presented in Plate VII. As seen in the Plate, test tubes in 'A' show the beginning of the screening while test tubes in 'B' show the reaction after 2 weeks. The dense yellow colouration indicates a positive result caused by the hydrolysis of pNPA to pNP by the bacterial isolates can be visibly seen in 'B' while the control remaining unchanged.

Plate VII: Test tubes used for the confirmatory test for CA production.
A: Day 1 of inoculation, B: After 2weeks of incubation.

4.1.1.2 Characterized and identified carbonic anhydrase producing bacteria

The two bacterial isolates GA (A) and GA (B) screened for CA production, GA(B) was found to produce crude enzyme extract with a significant CA activity, while the crude extract of isolate GA (A) showed minimal CA activity. Isolate GA (B) in addition to its morphological and biochemical characterization was further identified to molecular level, while the GA (A) was identified morphologically and biochemically, and the summary of results presented in Tables 4.1 and 4.2.

Table 4.1: Morphological and microscopic examination of carbonic anhydrase producing bacterial isolates

OBSERVATION	BACTERIAL ISOLATES	
	GA (A)	**GA (B)**
Form	Circular	Circular
Size	Small	Medium
Texture	Mucoid	Mucoid
Colour	Yellow	Orange-peach
Elevation	Raised	Flat

Margin	Entire	Entire
Microscopy	Bacilli	Bacilli

Table 4.2: Biochemical characteristics of carbonic anhydrase producing bacterial isolates

Test	GA (A)	GA (B)
Gram's reaction	+	-
Shape	Rod	Rod
Motility	+	+
Catalase	-	+
Citrate	-	-
Coagulase	-	-
CA Production	+	+
Indole	-	-
Oxidase	-	+
Vogues Proskauer	+	+
Spore	+	-
Hydrogen Sulphide Production	-	-
Anaerobic Incubation	+	-
Carbohydrate Utilization:		

	Ap	Gp	Ap	Gp
Glucose	+	-	-	-
Fructose	+	-	+	-
Arabinose	+	-	-	-
Maltose	+	-	+	+
Lactose	+	-	+	-

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

4.1.1.2.1 Molecular identity of isolate GARIMA (B) (GA (B))

The bacterial isolate GA (B) used revealed 99.9% similarity to *Alcaligenes faecalis* subsp. *parafaecalis* strain G after molecular characterization and identification. The image of the Agarose gel electrophoresis to the PCR amplified DNA of the bacterial isolate indicating a positive amplification of the 16S region of the bacteria isolate using 16S ribosomal universal primer is presented in Plate VIII.
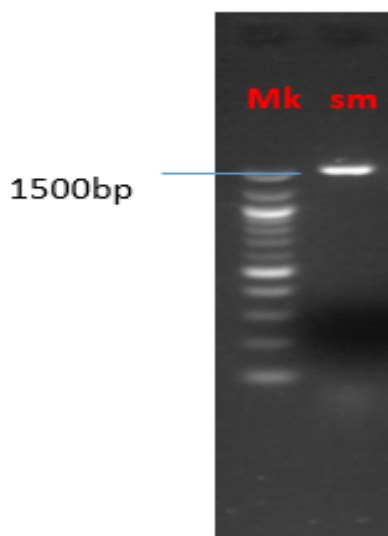

Plate VIII: Image showing the Agarose gel electrophoresis to PCR amplified DNA
Key: Mk= DNA Ladder 1500 bp, sm= Sample (*Alcaligenes faecalis* subsp. *parafaecalis* strain G)*, Organism's approximate size = 1500.

4.1.1.2.2 Partial sequence of *Alcaligenes faecalis* subsp. *parafaecalis* Strain G

The Gene sequenced obtained from the molecular characterization and identification revealed that the bacterial isolate GA (B) is 99% identical to *Alcaligenes faecalis* subsp. *parafaecalis* strain G, and the 16S ribosomal RNA gene is presented as:

MN847724 *Alcaligenes faecalis* subsp. *parafaecalis* strain G 16S ribosomal RNA, partial sequence GA (B)

TTTTAGTGGCGAGTGGCGGACGGGTGAGTAATATATCGGAACGTGCCCAGT
AGCGGGGGATAACTACTCGAAAGAGTGGCTAATACCGCATACGCCCTACGG
GGGAAAGGGGGGGATTCTTCGGAACCTCTCACTATTGGAGCGGCCGATATC
GGATTAGCTAGTTGGTGGGGTAAAGGCTCACCAAGGCAACGATCCGTAGCT
GGTTTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCC
TACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGGGAAACCCTGATCCA
GCCATCCCGCGTGTATGATGAAGGCCTTCGGGTTGTAAAGTACTTTTGGCAG
AGAAGAAAAGGTACCTCCTAATACGAGGTACTGCTGACGGTATCTGCAGAA
TAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAA
GCGTTAATCGGAATTACTGGGCGTAAAGCGTGTGTAGGCGGTTCGGAAAGA
AAGATGTGAAATCCCAGGGCTCAACCTTGGAACTGCATTTTTAACTGCCGA
GCTAGAGTATGTCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCG
TAGATATGTGGAGGAATACCGATGGCGAAGGCAGCCCCCTGGGATAATACT
GACGCTCAGACACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGT
AGTCCACGCCCTAAACGATGTCAACTAGCTCGTTGGGGCCGTTAGGCTTAGT
AGCGCAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGAAA
ACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAA
TTCGATGCAACGCGAAAACTTACCTACCCTTGACATGTCTGGAATGCCGAA
GAGATTTGGCAGTGCTCGCAAGARAACCGGAACACAGTGCTGCATGGCTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC
CTTTCATTAGTTGCTACGCAAGAGCACTCTAATGAGACTGCCGGTGACAAAC
CGGAGGAAGGTGGGGTGACTCAAGTCCTCATGGCCCTTATGGGTAGGGCTT
CACACGTCATACAATGGTCGGGACAGAGGGTCGCCAACCCGCGAGGGGGA
GCCAATCTCAGAAACCCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACT
GCGTGAAGTCGGAATCGCTAGTAATCGCGGATCAGAATGTCGCGGTGAATA
CGTTCCCGGGTCTTGTACACACCGCCCGTCACACCAAAAA.

4.1.1.2.3 Blast result of isolate GA(B)

The blast result for Isolate GA (B) showing the relationship with other organisms analysed is presented in the following Figure 4.1.
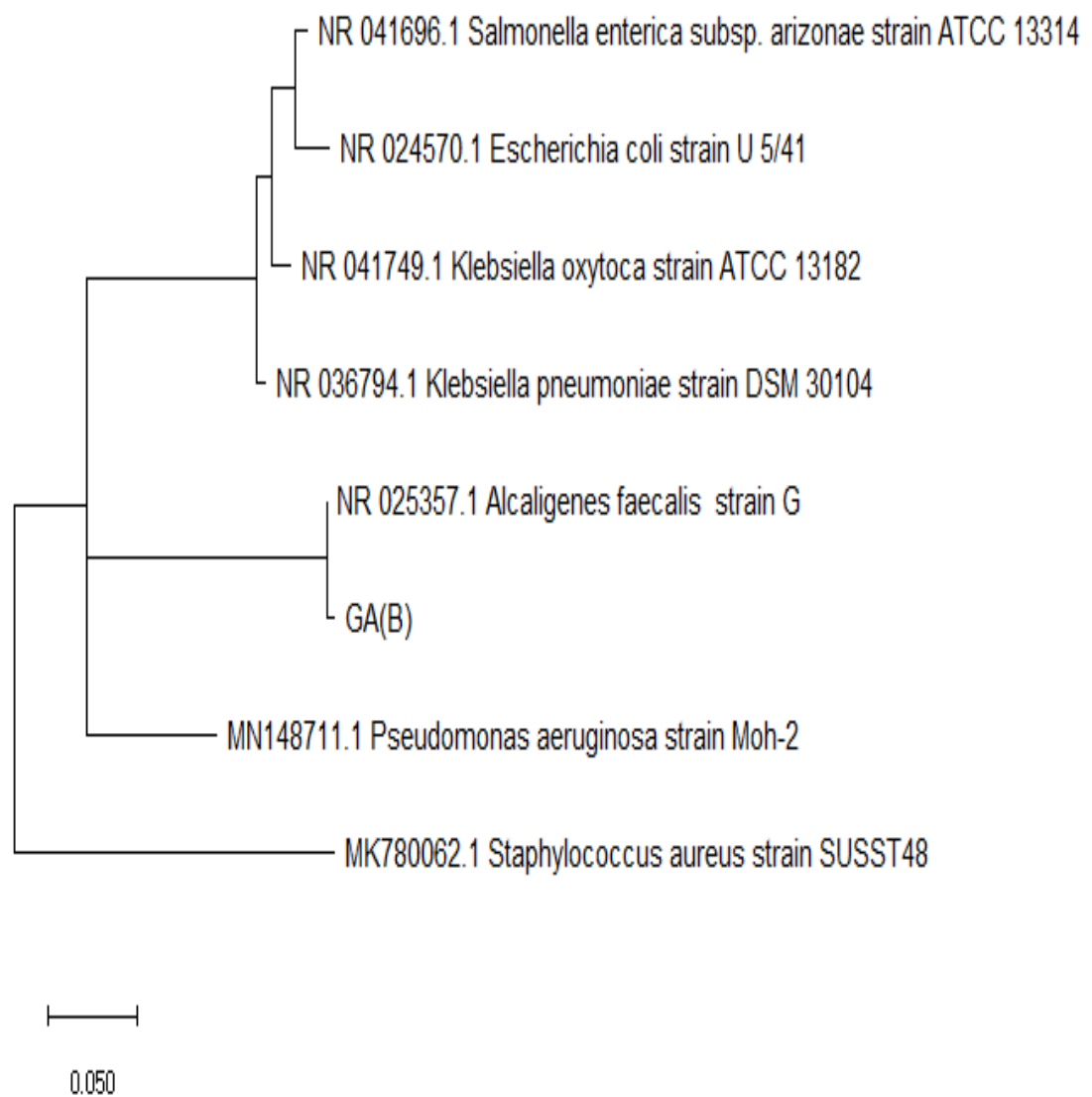
Figure 4.1: Blast results for Isolate GA (B)

4.1.2 Produced crude carbonic anhydrase

The two bacterial isolates GA (A) and GA (B) were used to produce crude carbonic anhydrase, and the result is presented in Plate IX.

Conical flask

Crude CA

Plate IX: Crude CA produced from the bacterial isolates

4.1.3 Carbonic anyhydrase activity results

The crude carbonic anhydrase (CA) extract from isolate GA(A) which had a mean enzymatic activity value of 0.03210 ± 0.0012 mmoles/mL^{-1}/sec^{-1}, while the CA activity of the crude CA extract obtained from *Alcaligenes faecalis* subsp. *parafecalis* strain G presented a higher enzymatic activity of 0.0351 ± 0.0002 mmoles/mL^{-1}/sec^{-1}. The following results in graphical presentation in Figure 4.2 were obtained for both isolates after statistical analysis.

Figure 4.2: Graphical representation of the CA activity for the 2 bacterial isolates

4.1.3.1 Optimum substrate concentration for CA activity

The substrate concentration was determined, and the following results as presented in
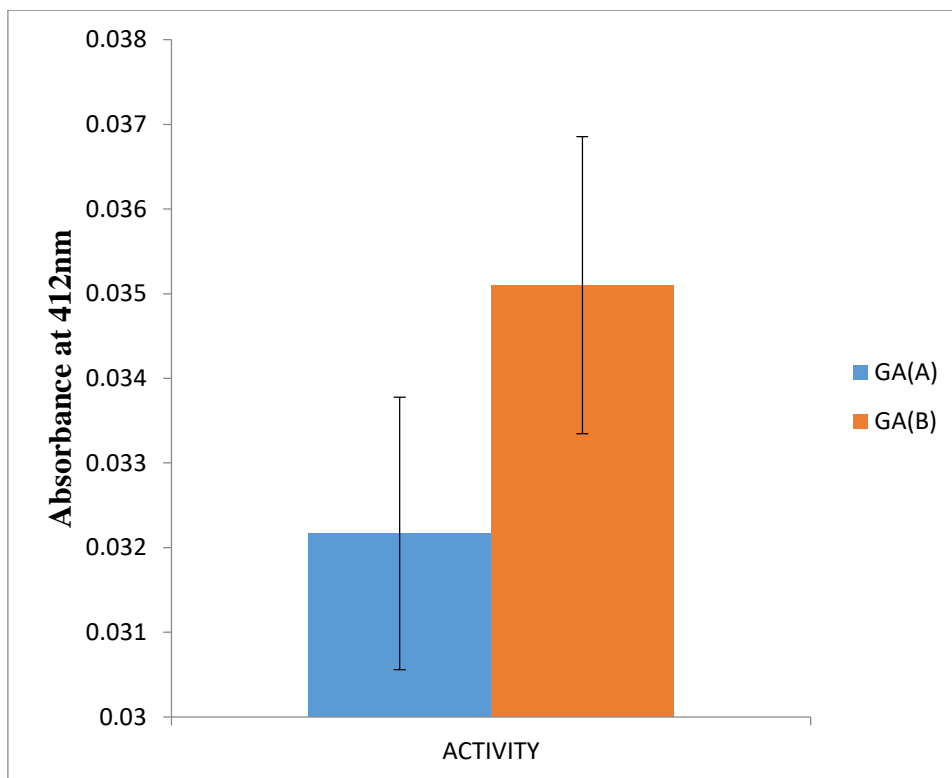
Table 4.3 were arrived at after statistical analysis.

Table 4.3: Mean enzyme activity for substrate optimization

Substrate concentration (mM)	GA(A)	GA(B)

1	$0.0115\pm2.3\text{X}10^{-4a}$	$0.0133\pm1.4\text{X}10^{-4a}$
2	$0.0224\pm1.7\text{X}10^{-4b}$	$0.0246\pm8.8\text{X}10^{-4b}$
3	$0.0321\pm1.4\text{X}10^{-4c}$	$0.0351\pm2.4\text{X}10^{-4c}$
4	$0.0433\pm1.4\text{X}10^{-4d}$	$0.0510\pm8.7\text{X}10^{-4d}$
5	$0.0440\pm1.4\text{X}10^{-4e}$	$0.0675\pm6.5\text{X}10^{-4e}$
6	$0.0433\pm1.4\text{X}10^{-4d}$	$0.0703\pm2.6\text{X}10^{-4f}$
7	$0.0436\pm1.5\text{X}10^{-4de}$	$0.0709\pm1.7\text{X}10^{-4f}$

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

For the crude CA extract from isolate GA (A), the results showed an increased CA activity with the substrate concentration from 1, 2 and 3 mM with their mean enzyme activity values with standard error of mean presented as 0.0115 ± 2.3 x 10^{-4}, 0.0224 ± 1.7 x 10^{-4}, and 0.0321 ± 1.4 x 10^{-4} mmoles/mL^{-1}/sec^{-1} respectively, with significant difference (P>0.05) between the results of enzyme activity. While for substrate concentrations 4, 5, 6 and 7 mM there mean enzyme activity with standard error of mean presented as 0.0433 ± 1.4 x 10^{-4}, 0.0440 ± 1.4 x 10^{-4}, 0.0433 ± 1.4 x 10^{-4}, and 0.0436 ± 1.4 x 10^{-4} mmoles/mL^{-1}/sec^{-1} respectively. There is no significant difference (P>0.05) between the mean enzyme activity of substrate concentration 4, 6 and 7 mM but there was significant difference between the mean enzyme activity values of substrate concentrations 4, 6 and 5 mM. Similarly, there was no significant difference in the mean enzyme activity values of substrate concentration 5 and 7 mM (at P>0.05).

The results of the mean enzymatic activity values of the crude CA extract from *Alcaligenes faecalis* subsp. *parafaecalis* strain G from substrate concentration 1 to 7 mM with standard error of mean are presented as 0.0133 ± 1.4 x 10^{-4}, 0.0246 ± 8.8 x 10^{-4}, 0.0351 ± 2.4 x 10^{-4}, 0.0510 ± 8.7 x 10^{-4}, 0.0675 ± 6.5 x 10^{-4}, 0.0703 ± 2.6 x 10^{-4}, and 0.0709 ± 1.7 x 10^{-4} mmoles/mL^{-1}/sec^{-1} respectively. The results showed significant

difference between the mean enzymatic activity values (at P>0.05) of substrate concentration 1 to 6 mM, while there was no significant difference between the mean enzyme activity of substrate concentration 6 and 7 mM (at P>0.05).

4.1.3.2 Optimum temperature for CA activity

The temperature conditions were varied from $30^{o}C-80^{o}C$ to determine the optimum temperature for the CA activity, and the results obtained are presented in the Table 4.4 below.

Table 4.4: Mean enzymatic activity for temperature optimization

Temperature (^{o}c)	GA (A)	GA (B)
30	$0.0246\pm8.82\times10^{-5b}$	$0.0290\pm8.82\times10^{-5b}$
40	$0.0304\pm8.82\times10^{-5c}$	$0.0316\pm1.15\times10^{-4c}$
50	$0.0329\pm1.15\times10^{-4d}$	$0.0370\pm1.45\times10^{-4e}$
60	$0.0306\pm1.45\times10^{-4c}$	$0.0350\pm6.08\times10^{-4d}$
70	$0.0306\pm1.2\times10^{-4c}$	$0.0314\pm3.48\times10^{-4c}$
80	$0.0194\pm8.82\times10^{-5a}$	$0.0244\pm3.33\times10^{-5a}$

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

For the crude CA extract from isolate GA(A), the results showed that there was significant difference (at P>0.05) in the mean enzymatic activity with standard error of mean from 30, 40 and $50^{o}C$ ($0.0246\pm8.82\times10^{-5}$, $0.0304\pm8.82\times10^{-5}$, and $0.0329\pm1.15\times10^{-4}$ mmoles/mL^{-1}/sec^{-1} respectively). Temperature range between 60 and $70^{o}C$ showed no significant difference in mean enzyme activity values with standard error of mean of $0.0306\pm1.45\times10^{-4}$ and $0.0306\pm1.2\times10^{-4}$ mmoles/mL^{-1}/sec^{-1} respectively. Temperature $80^{o}C$ has the least enzymatic activity with a mean enzyme activity with standard error of mean of $0.0194\pm8.82\times10^{-5}$ mmoles/mL^{-1}/sec^{-1} and differs significantly (at P>0.05) from all the other temperature values.

For the crude CA extract from *Alcaligenes faecalis* subsp. *parafaecalis* strain G, the results showed similarity in trends as obtained in GA (A). There was a significant difference in enzymatic activity (at $P>0.05$) from temperature 30-50°C with mean enzyme activity value with standard error of mean of $0.0290\pm8.82 \times 10^{-5}$, $0.0316\pm1.15 \times 10^{-4}$ and $0.0370\pm1.45 \times 10^{-4}$ mmoles/mL^{-1}/sec^{-1} respectively. Temperature 60-70°C showed no significant difference (at $P>0.05$) in enzymatic activity with mean enzyme activity values with standard error of mean of $0.0350\pm6.08 \times 10^{-4}$ and $0.0314\pm3.48 \times 10^{-4}$ mmoles/mL^{-1}/sec^{-1} respectively. At a temperature of 80°C, the mean enzyme activity with standard error of mean is $0.0244\pm3.33 \times 10^{-5}$ mmoles/mL^{-1}/sec^{-1}, differing significantly with the other values (at $P>0.05$).

4.1.3.3 Optimum pH for CA activity

Varying the pH from the range of 5.5-9.5, the optimum pH for CA activity of the two enzymes from the 2 bacterial isolates was determined and the results are presented in Table 4.5.

Table 4.5: Mean enzyme activity for pH optimization

pH	GA (A)	GA (B)
5.5	$0.0106\pm8.82\text{X}10^{-5a}$	$0.0119\pm8.82\text{X}10^{-5a}$
6.5	$0.0325\pm8.82\text{X}10^{-5b}$	$0.0337\pm1.12\text{X}10^{-3b}$
7.5	$0.0355\pm8.82\text{X}10^{-5c}$	$0.0355\pm1.10\text{X}10^{-3bc}$
8.5	$0.0357\pm8.82\text{X}10^{-5c}$	$0.0371\pm1.41\text{X}10^{-3cd}$
9.5	$0.0352\pm8.82\text{X}10^{-5c}$	$0.0385\pm1.04\text{X}10^{-3d}$
10.5	$0.0320\pm8.82\text{X}10^{-5b}$	$0.0337\pm1.2\text{X}10^{-4b}$

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

The results of the enzyme activity of crude CA extract from GA (A) showed that there was significant difference between the mean enzyme activity values with standard error of mean for pH 5.5 ($0.0106\pm8.82 \times 10^{-5}$ mmoles/mL^{-1}/sec^{-1}) and pH 6.5 ($0.0325\pm8.82 \times 10^{-5}$ mmoles/mL^{-1}/sec^{-1}), 10.5 ($0.0320\pm8.82 \times 10^{-5}$ mmoles/mL^{-1}/sec^{-1}), 7.5 (0.0355 ± 8.82

x 10^{-5} mmoles/mL^{-1}/sec^{-1}), 8.5 (0.0357±8.82 x 10^{-5} mmoles/mL^{-1}/sec^{-1}), 9.5 (0.0352±8.82 x 10^{-5} mmoles/mL^{-1}/sec^{-1}) (at $P>0.05$). pH 6.5 and 10.5 do not differ significantly, but they are significantly different from pH 7.5, 8.5 and 9.5 (at $P>0.05$). Similarly, there is no significant difference (at $P>0.05$) between pH 7.5, 8.5, and 9.5.

On the other hand, results obtained for the enzyme activity of crude CA extract from *Alcaligenes faecalis* subsp. *parafaecalis* strain G showed there was significant difference in the mean enzyme activity values with standard error of mean (at $P>0.05$) between pH 5.5 (0.0119±8.82 x 10^{-5} mmoles/mL^{-1}/sec^{-1}) and pH 6.5 (0.0337±1.12 x 10^{-3} mmoles/mL^{-1}/sec^{-1}), 10.5 (0.0337±1.2 x 10^{-4} mmoles/mL^{-1}/sec^{-1}), 7.5 (0.0355±1.10 x 10^{-3} mmoles/mL^{-1}/sec^{-1}), 8.5 (0.0371±1.04 x 10^{-3} mmoles/mL^{-1}/sec^{-1}), and 9.5 (0.0385±1.04 x 10^{-3} mmoles/mL^{-1}/sec^{-1}). There is no significant difference (at $P>0.05$) between the mean enzyme activity values with standard error of mean of pH 6.5, 7.5 and 10.5, no significant difference (at $P>0.05$) between the mean enzyme activity values with standard error of mean between pH 7.5 and 8.5, and also no significant difference between the values of pH 8.5 and 9.5 (at $P>0.05$).

4.1.4 Produced concretes

The pictures of the cast concrete are presented in plates X (control) and XI (bioconcrete) below.

Plate X: Concrete Specimen (Control)

Plate XI: Concrete Specimen (Bioconcrete)

4.1.5 Compressive strength results

The cubes mass and compressive strength results obtained after curing and crushing of the concrete specimens for days 7, 14 and 28 are presented in Table 4.6. The results shows that there was no statistically significant difference (at $P>0.05$) in the mass of the cube and crushing strength with the control and crude carbonic anhydrase (CA) using ANOVA.

There was no statistically significant difference between CA and expected strength (at $P>0.05$) using student T-test at day 7 while significant difference was recorded at day 14 and 21.

Statistically, there was no significant difference in the mean cubes mass values with standard error of mean (SEM) of the concretes (at $P>0.05$) (8.63 ± 0.18 kg for day 7, 8.76 ± 0.08 kg for day 14 and 8.46 ± 0.12 kg for day 28) as analysed for the control specimen. But for the bioconcrete, there was significant difference in the mean cubes mass values with SEM at $P>0.05$ between day 7 and day 14 (7.84 ± 0.34 and 8.86 ± 0.11 kg respectively) and no significant difference in the mean cubes mass values with SEM (at

P>0.05) between day 7 and day 28 (7.84±0.34 and 8.16±0.11 kg respectively) and day 14 and day 28 (8.86±0.11 and 8.16±0.06 kg respectively).

4.1.5.1 Crushing strength

Table 4.6: Crushing Strength

Days	Crushing Strength (N/mm^2)	
	Control	CA
7	20.46±11.43[a]	11.54±4.09[a]
14	23.11±0.25[a]	15.52±0.52[ab]
28	22.53±1.2[a]	22.28±1.70[b]
Percentage Strength gained	**9.19%**	**48.29%**

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

There was no significant difference in the mean crushing strength values with standard error of mean (SEM) of the control concrete cubes (at P>0.05) between days 7, 14 and 28 (20.46±1.43, 23.11±0.25 and 22.53±1.2 N/mm^2 respectively) as presented in Table 7 above. There was no significant difference (at P>0.05) in the mean crushing strength values with SEM between days 7 and 14 (11.54±4.09 and 15.52±0.52 N/mm^2 respectively). Similarly there was no significant difference in the mean crushing strength values with SEM of days 14 and 28 (at P>0.05) of (15.52±0.52 and 22.28±1.70 N/mm^2 respectively). However, there was significant difference (at P>0.05) in the mean crushing strength value with SEM between days 7 and 28 (11.54±4.09 and 22.28±1.70 N/mm^2 respectively), representing an increase in crushing strength from days 7 to 28. In addition, the percentage strength gained over the 28-days period for the control concrete specimens was 9.19%, while that of the bioconcrete was 48.29%.

4.1.6 SEM micrographs of the precipitated CaCO$_3$

The concretes (both the control and the bio-concrete) were viewed using the scanning electron microscopy (EVO LS 10 CARL ZEISS, Germany) at 100 μm, and the result is presented in Plate XII:
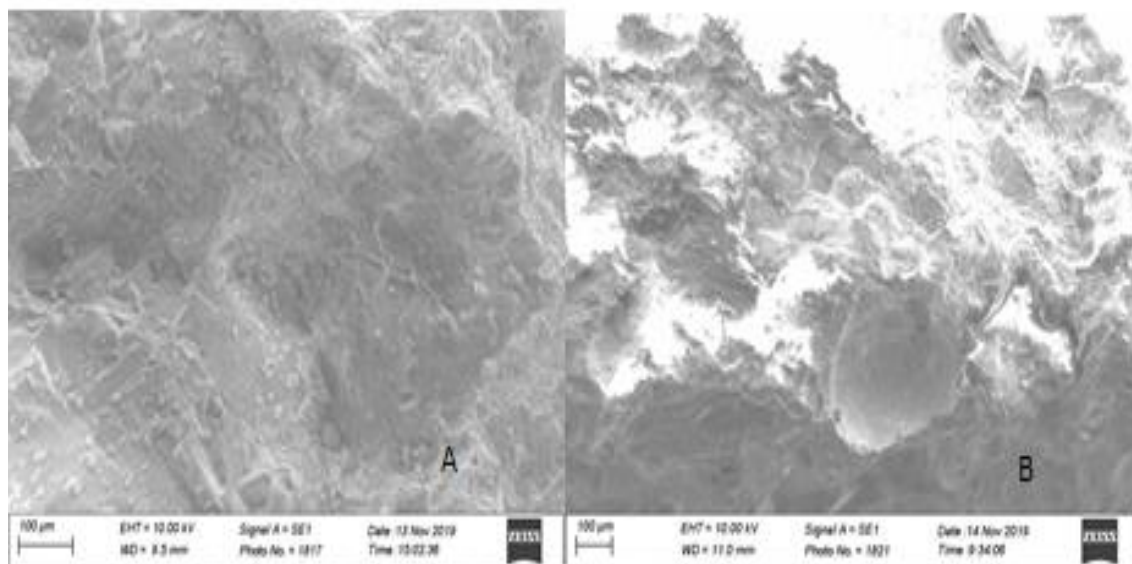
Plate XII: Scanning electron micrographs of the biocalcification showing A (control) and

B

 (bioconcrete).

As shown on the surface of the bioconcrete specimen (B), the visibly distinct white crystals of $CaCO_3$, which is absent on the control specimen (A). The distinctly visibly white crystals are precipitates of $CaCO_3$, resulting from the catalytic action of CA of hydrolyzing CO_2 into bicarbonate ion (HCO_3^-), which further reacted with available calcium (Ca^{2+}) to produce $CaCO_3$ thereby liberating hydrogen ion in the process.

4.2 Discussion

Carbonic anhydrase (CA) is a widely distributed enzyme in prokaryotes and known for their catalysis of the hydration of carbon (iv) oxide (CO_2) into carbonates. CA producing bacteria were isolated from soil samples from Garima and Kotangora construction sites around Bosso in Minna, Niger State. The two bacterial isolates showed the ability to produce carbonic anhydrase (CA), based on their ability to hydrolyse para-nitrophenylacetate (pNPA) to para-nitrophenol (pNP), thus producing distinct yellow colonies on the broth-peptone agar plates. In addition, observation was made on *Alcaligenes faecalis* subsp. *parafaecalis* strain G (GA (B)), which produced a peach/orange colouration, thus making it interesting for further studies.

The yellow colonies formation observed in isolate GA (A) is as a result of the hydrolysis of para-nitrophenylacetate (pNPA) by the bacterial produced CA, producing paranitrophenol (pNP) and acetate. The yellow colonies' reaction to pNPA is similar to the result reported by Li *et al*. (2004), who isolated carbonic anhydrase producing bacteria from the Karst region of China. On the other hand, the peach/orange colonies formation observed in *Alcaligenes faecalis* subsp. *parafaecalis* strain G, a Gram negative bacterium could be on one hand as a result of incomplete hydrolysis of the pNPA or as a result of the excessive hydrolysis of the pNPA by the bacterial CA. Even though there is contrasting colouration of the bacteria colonies, Alshalif *et al.,* (2018) did determine the ability of Gram negative bacteria to produce

CA using their ability to hydrolyse pNPA to pNP. This form of colonies colouration in reaction to pNPA has never been reported in literature, thus making it interesting for further studies even up to the genetic level.

Similarly, the reaction of the bacterial isolates in nutrient broth (presented in Plate V) to the supplemented pNPA to produce pNP and acetate, with the change in the colour of the nutrient broth from a light yellow to dense yellow colouration is as a result of the

hydrolysis of the pNPA by the two bacterial isolate GA (A) and *A. faecalis* subsp. *parafaecalis* strain G, while the control remained the same. This is a further indication that the two bacterial isolates produced CA which catalyses the hydrolysis of pNPA into pNP and acetate. This result conforms to similar result obtained by Alshalif *et al.* (2018), where the authors determined the production of CA by Gram negative bacteria isolates using their ability to hydrolyse p-NPA to produce p-NP.

These bacteria were characterized through morphological examination, microscopic observation and biochemical characterization. The isolate labelled GA (A) presented as a circular, small sized, mucoid, yellow, raised, and an entire colony, while isolate GA (B) appeared as a circular, medium sized, mucoid, orange-peach, flat, and with an entire colony. The microscopic observation, suggested that isolate GA (A) was Gram positive bacilli, while isolate GA (B) was Gram negative.

Molecular characterization of isolate GA (B) revealed that the isolate has 99% similarity to *Alcaligenes faecalis* subsp. *parafaecalis* strain G, a Gram negative bacterium that is non-spore forming but rather capsulated and positive to catalase and oxidase tests. Going by the unusual colouration (giving a peach/orange colour) in reaction to the pNPA hydrolysis to pNP, this has hitherto before now never been reported in any literature. Not many literature so far supports the production of CA by *A. faecalis* subsp. *parafaecalis* strain G, but with the results obtained from the activity of its crude enzyme produce showing a much higher activity in all the parameters tested, this has become an organism of interest for further study.

The carbonic anhydrase (CA) activity of the crude CA extract obtained from *A. faecalis* subsp. *parafecalis* strain G showed higher enzymatic activity of 0.0351±0.0002 mmoles/mL^{-1}/sec^{-1} when compared to the crude CA extract from isolate GA (A) which

had a mean enzymatic activity value of 0.03210 ± 0012 mmoles/mL^{-1}/sec^{-1}. The results indicate that the CA from *A. faecalis* subsp. *parafaecalis* strain G (GA (B)) had a slightly higher enzymatic activity when compared with GA (A). This feat could be attributed to a report in Basharat *et al.* (2018) which indicated that species in the genus *Alcaligenes* mostly, are able to withstand and degrade higher concentration of pollutants and heavy metals. There is a possibility however, of the over hydrolysis of the pNPA, which could explain the peach/orange colonies colouration observed during the screening with pNPA. For the crude CA extract obtained from isolate GA (A), the least enzymatic activity was recorded at substrate concentration 1 mM, while the highest activity was recorded at substrate concentration 5 mM. The optimum substrate concentration for optimum CA activity of GA (A) therefore is 5 mM at 0.0440 mmoles/mL^{-1}/sec^{-1}. The crude CA extract from *Alcaligenes faecalis* subsp. *parafaecalis* strain G presented a trending increase in enzymatic activity from substrate concentration 1 to 7 mM with mean enzyme activity values of 0.0133, 0.0246, 0.0351, 0.0510, 0.0675, 0.0703, and 0.0709 mmoles/mL^{-1}/sec^{-1} respectively. The results showed increased CA activity with increased substrate concentration. The crude CA extract obtained from *A. faecalis* subsp. *parafaecalis* strain G had the least enzyme activity at substrate concentration 1 mM (similar to that of GA (A)), while the highest enzyme activity was obtained at substrate concentration 7 mM, showing a direct correlation between substrate concentration and CA activity for crude CA extract from *A. faecalis* subsp. *parafaecalis* strain G. The optimum substrate concentration for optimum enzymatic activity of the crude CA extract from *A. faecalis* subsp. *parafaecalis* strain G was obtained at substrate concentration 7 mM with a mean enzyme activity value of 0.0709 mmoles/mL^{-1}/sec^{-1}.

The results for optimization of substrate concentration for optimum CA activity revealed contrasting enzymatic activity between the crude CA extract from *A. faecalis* subsp.

parafaecalis strain G and that of isolate GA (A), with optimum substrate concentrations of 7 mM and 5 mM respectively. The graph for both values showed that there is a similarity in the trend of sharp rise in the 2 curves from substrate concentration of 1 to 4 mM. while the crude CA from GA(A) maintained an almost steady plateau, that of *A. faecalis* subsp. *parafaecalis* strain G continued on an increase up till substrate concentration 5 mM after which the increase in activity was steady going in to substrate concentration 7 mM. The reason for the increased enzymatic activity with increased substrate concentration for the CA obtained from *A. faecalis* subsp. *parafaecalis* strain G could be as a result that most species in the genus *Alcaligenes* have been reported to be able to withstand and degrade higher concentration of pollutants and heavy metals (Basharat *et al.*, 2018), hence the inherent ability of the enzyme to have increased activity upon higher substrate concentration.

Temperature optimization for CA activity for the crude CA extract from GA(A), the results showed that that temperature 80°C has the least enzymatic activity with a mean enzyme activity with standard error of mean of 0.0194 mmoles/mL^{-1}/sec^{-1}, while the temperature with the highest CA activity is 50°C, it therefore means that the optimum temperature for CA activity of the crude CA extract from isolate GA (A) is 50°C with a mean enzyme activity value of 0.0329 mmoles/mL^{-1}/sec^{-1}. For the crude CA extract from *Alcaligenes faecalis* subsp. *parafaecalis* strain G, the results showed similarity in trends as obtained in GA (A). The least CA activity was recorded at temperature 80°C with a mean enzyme activity of 0.0244 mmoles/mL^{-1}/sec^{-1}, while the optimum temperature for optimum CA activity of the crude CA extract was 50°C with a mean enzyme activity value of 0.0370 mmoles/mL^{-1}/sec^{-1}.

The result closely conform to that obtained by Muley *et al.* (2014) who reported an enzyme activity of 0.0362 at a temperature of 50°C when working on the sequestration of

atmospheric CO_2 by microbial CA using *Bacillus schlegelii*. The result is in contrast to that of Littlechild (2015), who reported that CA from *Thermovibrio ammonificans* retained its activity after incubation for 1 hour at 70°C. The disparity in optimum temperature between the CAs from the two different bacteria is best explained considering the fact that the bacterium *T. ammonificans* is a deep sea inhabiting anaerobic, thermophilic, chemolithotrophic, and nitrate-ammnonifying bacterium. While the *A. faecalis* subsp. *parafaecalis* strain G on the other hand is a mesophilic bacterium commonly found inhabiting the environments such as soil and water. Similarly, the result is in contrast with the work of Demir *et al.* (2001), who reported an optimum temperature for human erythrocyte CA activity at 35°C. This is true, considering the fact that most mammalian optimum temperature is put at 37°C. There was also a slight difference in the result obtained by Demir *et al.* (2009) who worked on CA from potato (*Solanum tuberosum)* roots and leaves, and reported an optimum temperature of 40°C for CA activity from both the roots and leaves. However, the result showed similarity with that obtained by Li *et al*. (2015) who reported the identification of a new thermostable and alkali-tolerant α-CA from *Lactobacillus delbrueckii*. The authors reported that the α-CA was extremely stable at temperature 30°C-60°C, and the activity was maintained even beyond 90°C for 15 minutes.

pH optimization for CA activity was carried out and the optimum pH for CA activity for the CA produced by isolate GA (A) was obtained at pH 8.5 with a mean enzyme activity of 0.0357 mmoles/mL^{-1}/sec^{-1}. On the other hand, results for the enzyme activity of crude CA extract from *Alcaligenes faecalis* subsp. *parafaecalis* strain G showed similarities with that of GA (A) but with different optimum pH for optimum CA activity. The least enzymatic activity was obtained at pH 5.5, a result similar to the one obtained for GA (A). The optimum pH for CA activity of the crude CA extract from *A. faecalis* subsp.

parafaecalis strain G was obtained at pH 9.5, with mean enzyme activity of 0.0385 mmoles/mL^{-1}/sec^{-1}. This result is almost similar to the results obtained by Muley *et al.* (2014), who reported an enzyme activity of 0.0386 at optimum pH 10.0 when working on the sequestration of atmospheric CO_2 from microbial CA using *B. schlegelii.*.

The result is in contrast with the work carried out by Demir *et al.* (2001), where they obtained an optimum pH range value of 6.5-7.5 for CA activity from human erythrocyte which is the same for CA activity obtained from bovine erythrocyte plasma membrane. This is possible as a result of organismal variation, as humans and bovine are multicellular while the *A. faecalis* is unicellular. Also, Demir *et al.* (2009) in a work carried out on CA from potato (*Solanum tuberosum*) reported an optimum pH for CA activity to be closely conforming at 8.5 in the potato leaves and 11 in the roots. However, the results is in conformation with the work of Li *et al.* (2015) who reported a thermostable and alkaline-tolerant α-CA that was extremely stable at pH 6.0-11.0.

During the concrete production, with the concrete cubes cast, moulded and allowed to dry, an observation was made on the concrete cubes specifically the ones made with the crude CA extracts (bioconrete), as it took longer time to get hardened when compared to the concrete cubes without the extract (control). The extended hardening time for the bioconcrete was about six (6) hours, suggesting that there are activities going on in the concrete caused by the addition of the crude CA. In addition, the CA elicits a hydration process thereby keeping the concrete cubes extra hydrated, which could have extended the hardening time.

The mass of the concrete cubes for the control concrete specimens showed an increase from day 7-14, and there was a decrease from day 14-28. Similarly, the bioconcrete followed the same pattern of increase from day 7-14 and decrease from day 14-28. This could be as a result of the quality of the water used for curing and also the use of the same

tank for curing of the two concrete specimens. In addition, it could also be attributed to the fact that the CA in the bioconcrete upon immersion in water for curing and after exhausting the dissolved inorganic carbon lacked enough CO_2 to continue the calcite formation.

For the bioconcrete, the crushing strength increased from days 7 through 14 and 28 with mean crushing strength values of 11.54, 15.52 and 22.28 respectively. This result is similar to the one obtained by Lagazo *et al.* (2019) who reported similar pattern of crushing strength results increasing from days 7 through 14 and 28 of 14.89, 16.42 and 19.26 respectively, using the bacterium *B. subtilis* for concrete strengthening. In addition, the graph followed similar pattern, steadily progressing from the 7th through the 14th day. Similarly, in a result reported in Maheswaran *et al.* (2014) where they used a new wild type strain of *B. cereus* for strength improvement of cement mortar; using two (2) strains of *B. cereus* namely the microbial type culture collection (MTCC) and CSIR Chennai-India campus obtained strain (CS-I). Compressive strength results obtained from the MTCC strain for the 7th, 14th and 28th days in various cellular concentrations of 10^5 cell/ml of 28.65, 36.43 and 46.88 respectively, and 10^7 cells/ml of 33.0, 40.58 and 41.19 respectively. For the CS-I strain, the compressive strength obtained in various cellular concentrations of 10^5 cell/ml was for the 7th, 14th and 28th day was 25.65, 32.80 and 43.47 respectively; 10^6 cell/ml for the 7th, 14th and 28th day was 31.41, 38.80 and 50.52 respectively; and 10^7 cell/ml for the 7th, 14th and 28th day was 34.90, 38.32 and 44.00 respectively. The results reported by the authors revealed a similar growing trend in the graph curve when compared to the one obtained for the CA from *Alcaligenes faecalis* subsp. *parafaecalis* strain G enhanced concrete.

The increase in crushing strength in the bioconcrete was observed to be slow possibly as a result of the curing method employed. The immersion method of curing employed could

have largely affected the hardening and strength, giving that CA sequesters atmospheric CO_2, and upon utilization and exhaustion of the dissolved inorganic carbon present in the curing tank over time, the CA had limited or no CO_2 or the formation of calcium carbonate ($CaCO_3$).

The percentage strength gained over the 28-days period taken by comparing the strength at day 28 with the day 7 revealed that for the control concrete specimens, the percentage strength gain was 9.19%, while that of the bioconcrete was 48.29%. This implies that analysing the two different concretes comparatively, the bioconcrete with a 42.29% strength gain over 28 days showed an appreciable strength improvement when compared relatively to the 9.19% strength gain of the control concrete cubes.

Scanning electron micrographs of both the control and bioconcrete revealed obvious differences as shown in plate XII. The control concrete specimen (A) showed no visible precipitates of $CaCO_3$, whereas the bioconcrete (B) had visible $CaCO_3$ precipitates $CaCO_3$ crystals on the surface when viewed under a resolution of 100 μm. These visible precipitates of $CaCO_3$ crystals are a result of the reaction involving bicarbonate and calcium ions, catalysed by CA. This confers on the bioconcrete improved strength and also the ability to self-heal from cracks. These $CaCO_3$ are stable thermodynamically, eco-friendly and not readily water soluble. They are able to fill in the micro-cracks in concrete, affording them some level of compactness and acting as a sealant to the cracks, an indication that CA is a highly innovative tool for biocalcification of concrete. Similar result was reported in Maheswaram *et al.* (2014) and Anjana *et al.* (2015), where distinct precipitates of $CaCO_3$ were visibly presented in the SEM micrographs of the control and bacterially treated concrete.

CHAPTER FIVE

5.0   CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Carbonic anhydrase (CA) producing bacteria were isolated from concrete soil sites and further screened for their CA production ability. The two bacterial isolates were able to cause the hydrolysis of para-nitrophenylacetate (pNPA) into paranitrophenol (pNP) and acetate, producing distinct yellow and peach/orange colouration. Of the 2 bacterial isolates GA (A) and GA (B) capable of producing CA, the later was found to produce CA with higher enzyme activity. Molecular identification of the isolate GA (B) revealed that it was *Alcaligenes faecalis* subsp. *parafaecalis* strain G.

The CA produced by *A. faecalis* subsp. *parafaecalis* strain G was applied in the biocalcification of concrete and it showed considerable improvement in strength when tested for compressive and crushing strength, and with a percentage strength gain of 42.29% as compared to the control with a percentage strength gain of 9.19%. Scanning electron micrographs also revealed packets of precipitated calcium carbonate on the concrete surfaces. With the results obtained in the study, the CA could have huge potential applications in the biocalcification and healing of concrete.

5.2 Recommendations

The following recommendations are made after the research study, to help improve and guide future work on this research;

i. The CA produced from *Alcaligenes faecalis* subsp. *parafaecalis* strain G is recommended for application in concrete for biocalcification and crack healing.

ii. Employing other methods of curing that allows for exposure to atmospheric CO_2 such as membrane curing, cloth curing, shading curing, or the conventional water spraying curing method could aid the hardening and strength of the bioconcrete.

iii.   Genetic engineering and modification of the identified CA producing *A. faecalis* subsp. *parafaecalis* strain G should be considered so as to improve its ability to produce CA with a higher enzymatic activity and efficiency.

iv.   Increased concentration of the crude CA in concrete should be considered; concentration of CA:water of 90:10, 95:5 and even 100% CA concentration, instead of the 80:20 used for this research.

v.   Combined application of the crude CA with other possible enzymes should be explored to see the combined effect on concrete strengthening.

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APPENDIX A

MEDIA USED

1. Nutrient Agar

Lab-lemco powder	1.0 g
Yeast extract	2.0 g
Peptone	5.0 g
Sodium chloride	5.0 g
Agar (number 3 Oxoid)	15 g

Preparation was carried out based on manufacturer's instruction of 28 g to a liter of distilled water. For the purpose of this study, about 5.6 g of nutrient agar was measured in 200 ml of distilled water for every preparation, then autoclaved for 15 minutes at a temperature of 121ºC.

2. Broth-Peptone Agar

Peptone	10 g
Agar-Agar	15 g
Nutrient Agar	28 g

CaCO$_3$ 6.0 g

The preparation made for this study was for 100 ml of broth-peptone agar, and at every preparation 1 g of peptone, 1.5 g of agar-agar, 2.8 g of nutrient agar and 0.6 g of CaCO3 was measured into 100 ml of distilled water. The mixture was then dissolved and autoclaved for 15 minutes at a temperature of 121ºC.

3. Nutrient Broth (Oxoid)

Peptic digests of animal tissue	5.0 g
Sodium chloride (NaCl)	5.0 g
Beef extract	1.5 g
Yeast extracts	1.5 g
pH	7.4±0.2 at 25ºC

For the purpose of this study, 2.6 g of NB was measured into 200 ml of distilled water, the mixtures was thoroughly homogenized and autoclaved for 15 minutes at a temperature of 121ºC.

4. Sugar Fermentation Test Broth

Phenol red indicator	0.04 g
Peptone water (merck)	2.0 g
Distilled water	100 ml

For the purpose of this study, 100 ml of distilled water was used to dissolve the phenol red and the peptone water, and then autoclaved for 15 minutes at a temperature of 121ºC with the Durham's tubes (inverted) inside the mixture. After which 10 ml of the filtered sterilized sugars dissolved in sterile distilled water and heated over a hot burner were added.

5. Methyl-Red and Vogues-Proskuer Media

Peptone	5 g
Di-Potassium hydrogen phosphate (K$_2$HPO$_4$)	5 g

Glucose	5 g
Distilled water	100 ml
pH	7.5

For the purpose of this study, 15 g of the media was dissolved into a 100 ml of distilled water in a conical flask, and subsequently about 2 ml of the dissolved mixture was dispensed into test tubes and autoclaved for 15 minutes at a temperature of 121°C.

6. **Simmons Citrate Agar (Biomark ^{TM})**

Ammonium di-hydrogen phosphate	1.0 g
Di-potassium phosphate	1.0 g
Sodium chloride (NaCl)	5.0 g
Sodium citrate	2.0 g
Magnessium sulphate	0.2 g
Agar	15 g
Bromothymol blue	0.08 g
Distilled water	1000 ml
pH	
6.9±0.	

7. **Motility Test Media**

Peptone water (merck)	2.0 g
Sodium chloride (NaCl)	1.5 g
Agar agar (Oxoid)	2.5 g

Distilled water   100 ml

pH   7.1±0.2

For the purpose of this study, the 6.5 g composition was dissolved in 100 ml of distilled water and then dispensed into test tubes (of 5 ml each), it was then autoclaved for 15 minutes at a temperature of 121°C.

8. Fermentation Medium

Sucrose	5.0 g
Di-Sodium Hydrogen Phosphate (Na_2HPO_4)	2.0 g
Magnessium Sulphate ($MgSO_4.7H_2O$)	0.5 g
Calcium Carbonate ($CaCO_3$)	0.1 g
Iron (iii) chloride ($FeCl_3.6H_2O$)	0.005 g
Ordinary Portland Cement	5.0 g
Distilled water	1000 ml

For the purpose of this study, and for every preparation made, the mixture was thoroughly shaken to allow for homogenization, and then autoclaved for 15 minutes at a temperature of 121°C.

APPENDIX B

REAGENTS USED

1. Benedict's Solution

Sodium citrate	17.3 g
Na_2CO_3 anhydride	10 g
$CuSO_4.5H_2O$	1.73 g
Distilled water	100 ml

2. Kovac's Reagent

P-dimethylaminobenzaldehyde	5.0 g

| Amy alcohol (95%) | 75 ml |
| Conc. HCl | 25 ml |

3. Crystal violet

| Crystal violet powder | 0.5 g |
| Distilled water | 100 ml |

4. Safranine

Safranin powder	2.5 g
Ethyl alcohol (95%)	100 ml
Distilled water	900 ml

5. Methyl red solution

Methyl red	0.04 g
Absolute ethanol	40 ml
Distilled water	100 ml

6. Oxidase reagent

1% tetramethyl-p-phenylenediamine aqueous solution

7. Para-Nitrophenylacetate (pNPA)

pNPA	10g
Acetone	50 ml
Distilled water	100 ml

APPENDIX C

RAW DATA

Enzyme activity of the Carbonic Anhydrases from isolates GA (A) and GA (B) at 412 nm absorbance

GA(A)			GA(B)		
1	2	3	1	2	3
0.0324	0.0321	0.032	0.0346	0.0352	0.0355

Blank absorbance at 412 nm using para-nitrophenol (pNP)

Concentration(mg/L)	Wavelength(nm)	
1	412	0.080
2	412	0.151
3	412	0.208
4	412	0.241
5	412	0.303

Mean enzyme activity of the CAs from isolates GA(A) and GA(B)

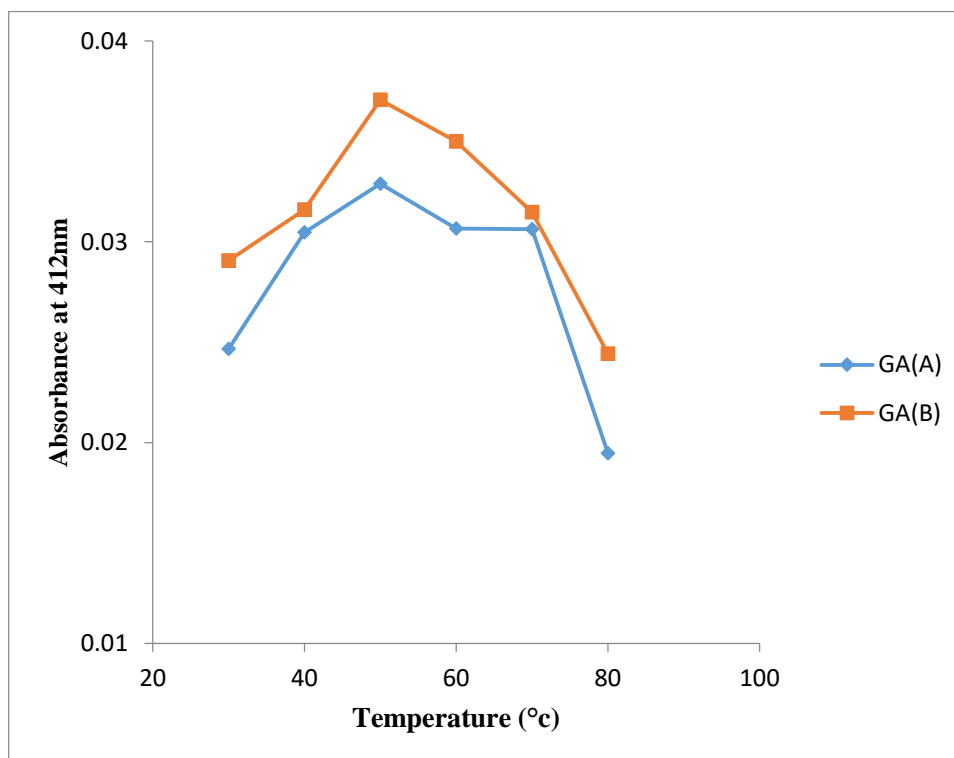| BACTERIA	ACTIVITY
GA(A)	0.0321±0.0012
GA(B)	0.0351±0.0002

Mean enzyme activity with standard error mean (±SEM)

Temperature Optimization for CA Activity for Isolates GA(A) and GA(B)

	GA(A)			GA(B)		
Temperature (^{o}C)	1	2	3	1	2	3
30	0.0245	0.0247	0.0248	0.0289	0.0292	0.0291
40	0.0303	0.0306	0.0305	0.0314	0.0318	0.0316

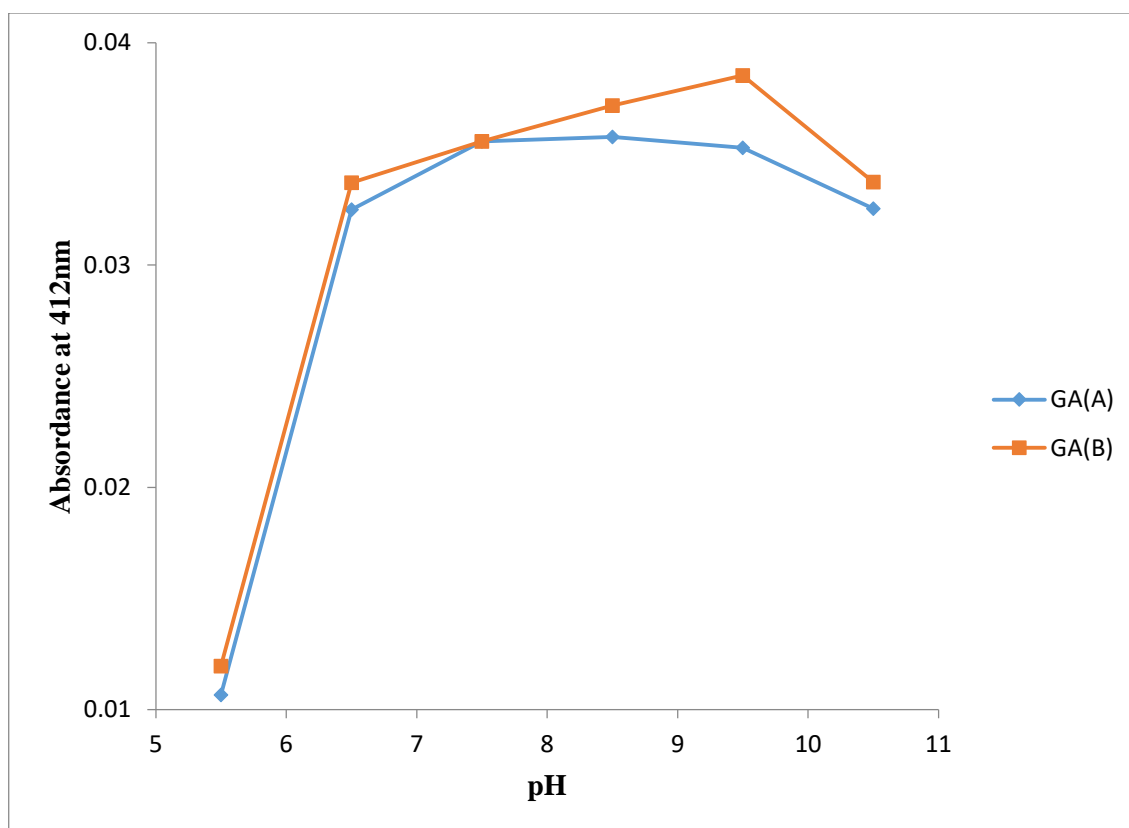|	1	2	3	1	2	3
50	0.0327	0.0331	0.0329	0.0368	0.0371	0.0373
60	0.0304	0.0309	0.0307	0.0339	0.0351	0.036
70	0.0304	0.0308	0.0307	0.0309	0.0321	0.0314
80	0.0193	0.0196	0.0195	0.0244	0.0245	0.0244

Graphical representation of the optimum temperature for CA activity for the 2 bacterial isolates

pH Optimization for CA Activity of Isolates GA(A) and GA(B) at 412 nm absorbance

	GA(A)			GA(B)		
pH	1	2	3	1	2	3
5.5	0.0105	0.0108	0.0107	0.0118	0.012	0.0121

| 6.5 | 0.0291 | 0.0344 | 0.0342 | 0.0315 | 0.0349 | 0.0347 |
7.5	0.0356	0.0354	0.0357	0.0356	0.0353	0.0358
8.5	0.0356	0.0359	0.0358	0.0358	0.04	0.0357
9.5	0.0351	0.0353	0.0354	0.0367	0.0403	0.0386
10.5	0.0324	0.0327	0.0325	0.0335	0.0339	0.0338

Optimum pH for CA activity for the 2 bacterial isolates

Substrate Optimization for CA Activity of Isolates GA(A) and GA(B) at 412 nm Absorbance

Substrate conc.(mM)	GA(A)			GA(B)		
	1	2	3	1	2	3
1	0.0111	0.0119	0.0116	0.0131	0.0136	0.0134
2	0.0221	0.0225	0.0227	0.0248	0.0245	0.0247
3	0.0322	0.0324	0.0319	0.0347	0.0355	0.0353
4	0.0436	0.0431	0.0434	0.0493	0.0521	0.0517
5	0.0438	0.0441	0. 0443	0.0685	0.0663	0.0679
6	0.0433	0.0431	0.0436	0.0708	0.0702	0.0699
7	0.0437	0.0433	0.0438	0.0706	0.0712	0.0709

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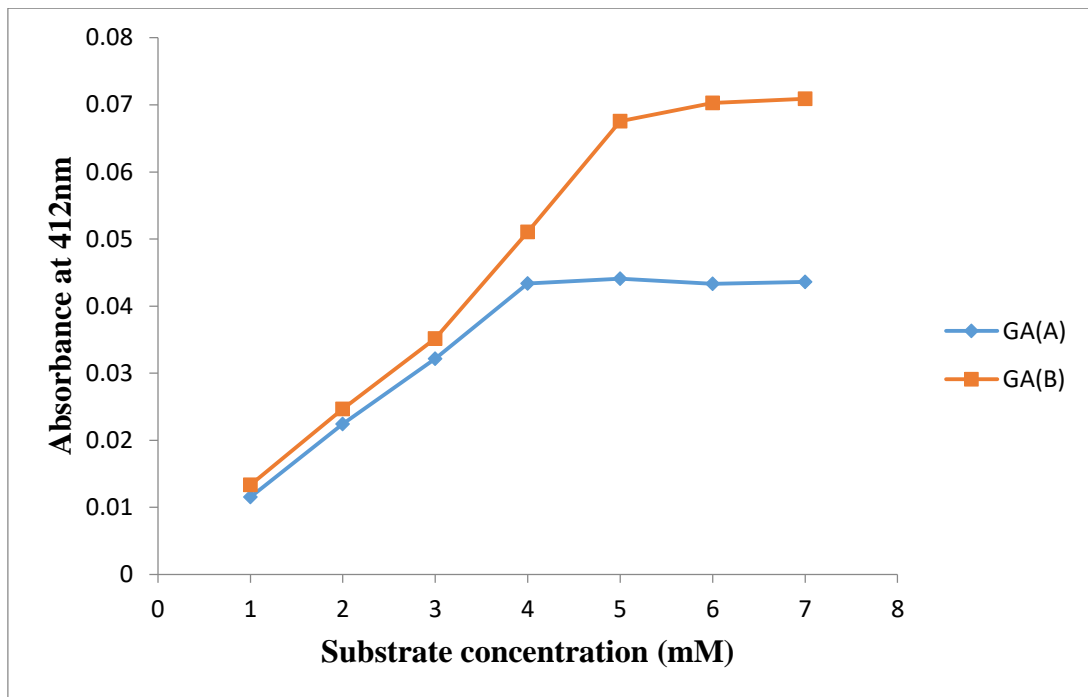
Graphical representation of the substrate concentration for CA activities of the 2 bacterial isolates
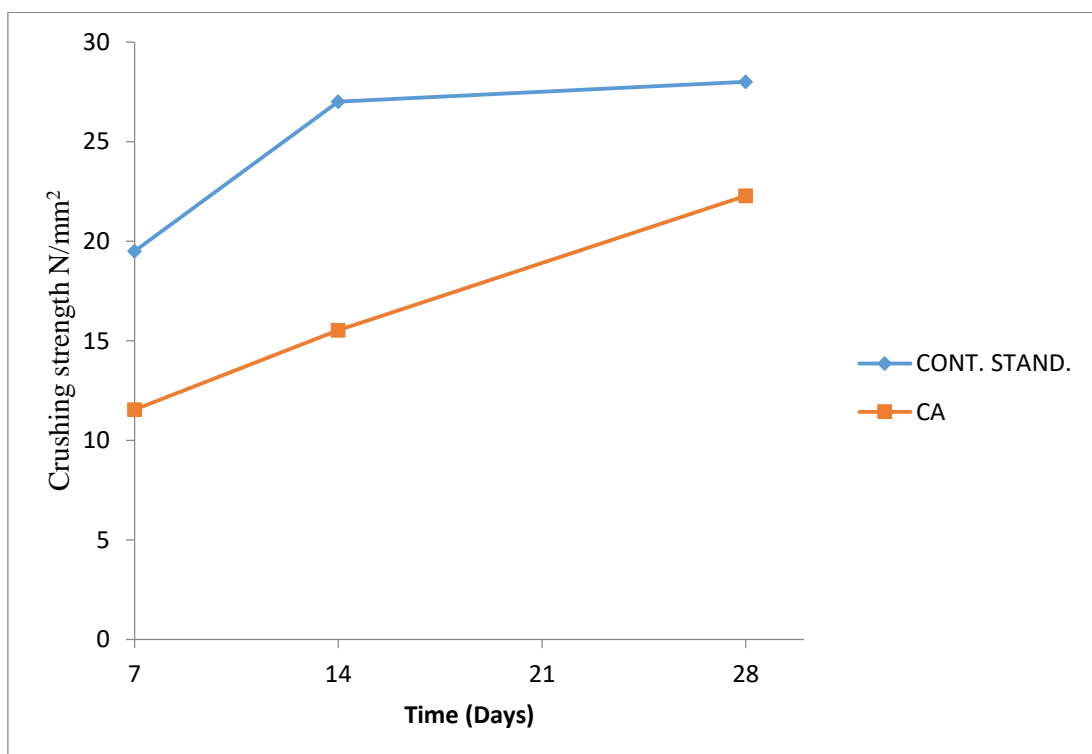
Graphical representation of the crushing strength of the bioconcrete and control

The compressive strength raw results

- A' 7days

CONCRETE GRADE	30
CONCRETE MIX RATIO (GRAVIMETRIC):	1 : 1.5: 3
CONCRETE MIX RATIO (VOLUMETRIC):	1 : 2 :4
CEMENT:	ORDINARY PORTLAND CEMENT
CURING METHOD:	IMMERSION IN WATER
MOULD SIZE/VOL:	150mm x 150mm x 150mm = 0.003375 m^3
MOULD LOAD BEARING AREA:	150mm x 150mm = 22,500 mm^2

Cube No	Casting Date	Testing Date	Age (Days)	Mass of Cube(Kg)	Density (Kg/m3)	Crushing Load(KN)	Crushing Strength(N/mm3)

1	3/10/2019	11/10/2019	7	8.40	2488.89	409	18.18
2	3/10/2019	11/10/2019	7	8.50	2518.52	452	20.09
3	3/10/2019	11/10/2019	7	9.00	2666.67	520	23.11

*Mean Strength: 20.46 N/mm^2 *Expected Strength: 19.50 N/mm^2 *Ratio/Result: 1.05/Pass

Expected Strength

1 day	16%	4.80	N/mm^2
3 days	40%	12.00	N/mm^2
7 days	65%	19.50	N/mm^2
14 days	90%	27.00	N/mm^2
28 days	99%	29.70	N/mm^2

CA _7days

CONCRETE GRADE	30
CONCRETE MIX RATIO (GRAVIMETRIC):	1 : 1.5: 3
CONCRETE MIX RATIO (VOLUMETRIC):	1 : 2 :4
CEMENT:	ORDINARY PORTLAND CEMENT

CURING
METHOD: IMMERSION IN WATER
MOULD
SIZE/VOL: 150mm x 150mm x 150mm =   0.003375   m³
MOULD LOAD BEARING AREA: 150mm x 150mm =   22,500   mm²

Cube No	Casting Date	Testing Date	Age (Days)	Mass of Cube(Kg)	Density (Kg/m3)	Crushing Load(KN)	Crushing Strength(N/mm3)
1	3/10/2019	11/10/2019	7	7.20	2133.33	160	7.11
2	3/10/2019	11/10/2019	7	7.92	2346.67	444	19.73
3	3/10/2019	11/10/2019	7	8.40	2488.89	175	7.78

*Mean Strength: 11.54 N/mm² *Expected Strength: 29.70 N/mm² *Ratio/Result: 0.39/Fail

A'_14days

CONCRETE
GRADE   30
CONCRETE   MIX   RATIO   1 : 1.5:
(GRAVIMETRIC):   3
CONCRETE   MIX   RATIO
(VOLUMETRIC):   1 : 2 :4

CEMENT:	ORDINARY PORTLAND CEMENT
CURING METHOD:	IMMERSION IN WATER
MOULD SIZE/VOL:	150mm x 150mm x 150mm = 0.003375 m³
MOULD LOAD BEARING AREA:	150mm x 150mm = 22,500 mm²

Cube No	Casting Date	Testing Date	Age (Days)	Mass of Cube(Kg)	Density (Kg/m3)	Crushing Load(KN)	Crushing Strength(N/mm3)
1	3/10/2019	18/10/2019	14	9.04	2678.52	344	15.29
2	3/10/2019	18/10/2019	14	8.92	2642.96	330	14.67
3	3/10/2019	18/10/2019	14	8.64	2560.00	374	16.62

*Mean Strength: 15.53 N/mm² *Expected Strength: 29.70 N/mm² *Ratio/Result: 0.52/Fail

A'_ 28days

CONCRETE GRADE	30
CONCRETE MIX RATIO (GRAVIMETRIC):	1 : 1.5: 3
CONCRETE MIX RATIO (VOLUMETRIC):	1 : 2 :4

CEMENT:	ORDINARY PORTLAND CEMENT
CURING METHOD:	IMMERSION IN WATER
MOULD SIZE/VOL:	150mm x 150mm x 150mm = 0.003375 m³
MOULD LOAD BEARING AREA:	150mm x 150mm = 22,500 mm²

Cube No	Casting Date	Testing Date	Age (Days)	Mass of Cube(Kg)	Density (Kg/m3)	Crushing Load(KN)	Crushing Strength(N/mm3)
1	3/10/2019	2/11/2019	28	8.6	2548.15	562	15.29
2	3/10/2019	2/11/2019	28	8.7	2577.78	677	14.67
3	3/10/2019	2/11/2019	28	8.5	2518.52	564	16.62

*Mean Strength: 26.71 N/mm² *Expected Strength: 19.50 N/mm² *Ratio/Result: 1.37/Pass

CA_ 28days

CONCRETE GRADE	30
CONCRETE MIX RATIO (GRAVIMETRIC):	1 : 1.5: 3
CONCRETE MIX RATIO (VOLUMETRIC):	1 : 2 :4
CEMENT:	ORDINARY PORTLAND CEMENT

CURING
METHOD: IMMERSION IN WATER
MOULD
SIZE/VOL: 150mm x 150mm x 150mm =   0.003375   m^3
MOULD LOAD BEARING AREA: 150mm x 150mm =   22,500   mm^2

Cube No	Casting Date	Testing Date	Age (Days)	Mass of Cube(Kg)	Density (Kg/m3)	Crushing Load(KN)	Crushing Strength(N/mm3)
1	3/10/2019	2/11/2019	28	8.30	2459.26	446	19.82
2	3/10/2019	2/11/2019	28	8.1	2400.00	575	25.56
3	3/10/2019	2/11/2019	28	8.1	2400.00	483	21.47

*Mean Strength: 22.28 N/mm^2 *Expected Strength: 29.70 N/mm^2 *Ratio/Result: 0.75/Fail