Role of microbial concrete in steel slag amended concrete structures

A DISSERTATION

Submitted in the partial fulfillment of the requirements for the award of the degree of

MASTERS OF SCIENCE

IN

BIOTECHNOLOGY

Under the supervision of

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CERTIFICATE

Certified that the thesis "**Role of microbial concrete in steel slag amended concrete structures**" which is submitted by **Ms. Prabhdeep Kaur**, in fulfillment of the requirement for the award of the degree of **Master of Science** in the **Department of Biotechnology**, Thapar University, Patiala, is a record of the candidate's own independent and original research work carried out by her under my supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other University or institute for the award of any degree.

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DECLARATION

I hereby declare that the work which is being presented in this thesis "**Role of microbial concrete in steel slag amended concrete structures**" submitted by me for the award of the degree of Master of Science in the **Department of Biotechnology**, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervision of **Dr. M. Sudhakara Reddy**, Professor, Department of Biotechnology, Thapar University, Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other University or institute for the award of any degree in India or abroad.

Date: 17-07-17
Place: PATIALA

[PRABHDEEP KAUR]

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ABSTRACT

Worldwide production of crude steel was a total of 1.63 billion metric ton in the year 2016. India being an emerging economy has a fast pace growth in industrial sector and is world's third largest steel producer. The high production of steel not only includes economy-based concerns but also have environmental concerns associated with it. Bulk generation steel slag which is waste byproduct generated from steel industries involves disposal crises. The cement industry faces a number of challenges that include scarcity of raw materials and a perpetually increasing demand for concrete. The use of steel slag a source of fine aggregate replacement in concrete is a way for safe and economical utilization of waste byproducts. Along with the replacement, durability enhancement of the concrete harvesting Microbially induced calcium carbonate precipitation (MICCP), which is a biomineralization process is exploited in the current study.

In the present study, calcifying bacteria PP9 isolated from steel slag was used to enhance the durability of cementitious material. Phylogenetic analysis of PP9 revealed 98.94% similarity to *Staphylococcus pasteuri.* The isolate PP9 was confirmed as non-pathogenic by coagulase test and mannitol salt agar. Urease assay quantitatively confirmed high urease production. Waste utilization was done by replacing fine aggregate in cement with steel slag (30% and 50%) along with MICCP carried out by isolated strain PP9. From the present investigation, it was clear that slag replacement along with bacterial treatment of motar cubes by PP9 effectively enhanced the durability and permeation properties. Compressive strength increased by 28% in bacterial treated specimen with 30% slag replacement (30SBT) when compared to the control motar cube. Water absorption property of 30SBT specimen decreased by 8 times when compared to the control. Scanning electron microscopy analysis further supported the involvement of bacteria in formation of calcite and vaterite polymorphs of calcium carbonate on cement motar cubes. Hence, bacterial treatment along with steel slag replacement in building materials proved to efficiently enhance its properties as well as diminish environmental related concerns.

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ABBREVIATIONS

°C	Degree Celsius
30CS	30% Slag replacement in cement specimen
30SBT	30% Slag replacement and Bacterial treated specimen
50CS	50% Slag replacement in cement specimen
50SBT	50% Slag replacement and Bacterial treated specimen
BF	Blast Furnace
BOF	Basic Oxygen Furnace
C	Control specimen
CNS	Coagulase Negative Staphylococcus
DNA	Deoxyribonucleic acid
EAF	Electric Arc Furnace
EDTA	Ethylenediamine Tetra Acetic acid
EDX	Energy Dispersive X-ray spectroscopy
Fig	Figure
hrs	Hours
kV	Kilo volt
M	Molar
mA	Mili ampere
MICCP	Microbially induced calcium carbonate precipitation
mins	Minutes
mL	Milli litre

mm	Mili metre
MSA	Mannitol salt agar
ng	Nano gram
OPC	Ordinary Portland Cement
PCR	Polymerase Chain Reaction
rDNA	Recombinant DNA
RFLP	Restriction fragment length Polymorphism
rpm	Revolution per minute
rRNA	Ribosomal RNA
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscope
XRD	X-ray diffraction
µ mole	Micro mole
µl	Micro litre

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CHAPTER 1

INTRODUCTION

Concrete has emerged as the most widely used construction material in the world since nineteenth century. Rapid urbanization resulted in its accelerated consumption. Its the first choice for buildings especially large scale constructions and infrastructure due to its various attractive properties which include high compressive strength, easy casting, fire resistance, durability under aggressive environments. However, the major drawback with concrete is its long-term performance and durability in non-ideal service environment. Severe degradation and deterioration of mechanical and structural properties occurs due to physical and chemical attack. This results in the overall reduction of the service life (De Belie et al., 2016).

The long-term performance of the concrete system is governed by the inter relationship of the environment, microstructure of the cement paste and the fracture strength of the concrete. The deterioration factors consist of seasonal temperature variations; freeze thaw cycles, relative humidity variations and concentration of deleterious chemicals in the ground. Irreversible changes in serviceability occur due to external environmental attacks like sulphate ingress, chloride ingress, and carbonation of concrete. Most of the factors that initiate and propagate the corrosion process are directly related to the permeability and mechanical strength of concrete (Basheer et al., 1996). Various surface treatments like acrylic coatings pore blockers; siloxanes and epoxy coatings are used to refine the pore structure of concrete in order to protect premature deterioration. These however are not preferred due to constant maintenance requirement, degradation with time and environmental impact (De Muynck et al., 2010).

The cement industry faces challenges such as depleting fuel reserves, raw materials and growing environmental concerns. Several advances have been made to address these concerns in the recent past. Hence concrete durability in a sustainable way becomes a hot research issue with the increasing concern of high CO_2 emissions during its production, high maintenance and repair costs.

In order to become sustainable, construction industry need to look into social concerns (health and well-being) environmental impact (material and energy use) and economic liabilities (construction cost). Elevated exhaustion of natural resources, immense amount of production of industrial wastes and environmental pollution require obtaining new solutions for a sustainable development by increasing the resource efficiency.
Concrete technology demands usage of huge amount of natural resources such as sand, gravel, cement and water. Around 3.5 billion tons of cement is used annually in the cement production in the world (https://cembureau.eu/). For this reason, using three criteria such as cost of materials and construction, durability and environment friendliness must develop concrete technologies.

Not only should building materials satisfy the performance needs of the user but also the development needs of the society without causing any adverse impact on environment (Reddy and Jagdish 2003). Most prevalent among them are blending cement with industrial byproducts and solid wastes such as fly ash, blast furnace slag, bottom ash, waste glass and silica fume etc. One of the fastest growing sectors of the manufacturing industry all over the world is stainless steel (Huaiwei et al., 2011).

Steel slag is generated as waste material or byproduct from steel industries. Per ton of steel during the steel production generates about 2–4 ton of wastes (Das et al., 2007; Devi et al., 2014). Simple disposals of these wastes are becoming less attractive since it not only occupies plenty of land but also increases the disposal costs. Natural weathering proceeds too slowly and different techniques like autoclaving consume high energy. Hence more sustainable bio geochemical inspired method needs to be considered to treat and replace the aggregates with slag in concrete. Steel slag can be used as a cementing component in several cementing systems. However, some material being able to consume free lime and to eliminate potential expansion must be used together with steel slag (Shi 2004).

Along with the replacement, durability enhancement of the concrete is considered to improve the concrete strength and life span. Microbial metabolic activities both intra- and extracellular often contribute to the selective cementation by producing relatively insoluble organic and inorganic compounds (Stock Fischer et al., 1999). Calcium

Carbonate bacterial mineralization has found applications in the remediation, consolidation and restoration of various building materials (Dhami et al., 2012a). Precipitation of the calcium carbonate crystals on the bacterial cell wall occurs by heterogeneous nucleation. These crystals precipitate inside the pore spaces once super saturation is achieved. The bacteria not only initiate calcite precipitation but also serve as nucleation sites for calcite crystals and association with many other factors like dissolved inorganic carbon, pH, Calcium ions and temperature in the medium (Hammes and Verstraete 2002, Stock Fishcer et al., 1999). These calcite crystals acts as cloggers by filling the voids and thereby reducing permeability in the building materials (Dhami et al., 2012b) In cement motar cubes bacterial calcite led to the enhanced compressive strength along with the reduced corrosion in reinforced concrete and reduction in water and chloride ion permeability (Achal et al., 2010, 2011, 2012).

All these observations indicated that bacterial precipitation proceeds as long as ingredients and nutrients for the microbial growth are available. Several different processes can accomplish the precipitation of carbonates. These including urea hydrolysis, sulphate reduction, dolomite precipitation and iron reduction **(**Ehrlich, 1998; Castanier et al., 1999). However, hydrolysis of urea presents several advantages over the other carbonate generating pathways. Few of them include its potential to produce high amounts of carbonates within a short period of time, energy efficient process and also easily controllable (DeJong et al., 2010). During the enzymatic urea hydrolysis, the enzyme urease causes an increase in pH that shifts the bicarbonate-Carbonate equilibrium towards the production of more carbonate ions and finally lead to the precipitation of $CaCO_3$ (Castanier et al., 2000).

Slag with the presence of high lime content also contributes to the calcium source for bacterial utilization. Hence, a concept of replacement of aggregate with steel slag waste in order to cut down on the cost and environmental impact of sand mining, as well as incorporation of bacterial calcium carbonate precipitation to enhance the compressive strength of the concrete is worked upon. Overall concept of replacement using waste steel slag and durability enhancement by exploiting the Microbially induced calcium carbonate precipitation (MICCP) property of bacterial species is experimented and studied further.

Objectives

- Isolation and identification of Ureolytic bacteria from steel slag
- Physiological and molecular characterization of bacteria
- Pilot studies on microbially treated cement motar cubes along with steel slag replacement (30% and 50%)

CHAPTER 2

REVIEW OF LITERATURE

2.1 Cement and its deterioration factors

In the world of construction, one material used above all is concrete. Concrete is far more produced all over the world than any other man made material. It is incredibly versatile, and is used in almost all major construction projects (Vasoya et al., 2015). Its various attractive properties include high compressive strength, easy casting, fire resistance and durability under aggressive environments (De Belie et al., 2016).

During the middle of the last century constructions took place in the developed world and they are facing the challenges of maintenance and renewal. The emerging economies, on the other hand, are rapidly building their infrastructure. As a result, consumption of building materials has grown at a very fast pace and would continue to grow in the time to come (Schneider et al., 2011). It's hard to imagine a world without concrete, and its dominant precursor, Ordinary Portland Cement (OPC). Cement industry is growing particularly rapidly in developing countries such as India and China that have a high demand for infrastructure and housing. Estimated production of cement worldwide is shown in Fig. 2.1 (Imbabi et al., 2012).

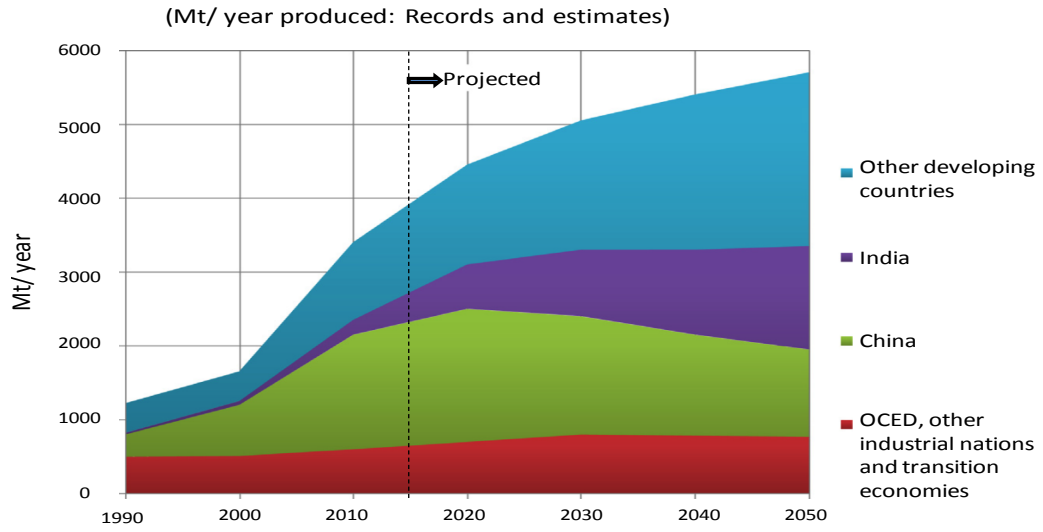


Fig. 2.1. World Portland cement production estimates 1990–2050. (Source: Imbabi et al., 2012)

Buildings are not only one of the largest consumers of natural resources but also they account for a significant portion of greenhouse gas emissions (Chen et al., 2010). The cement industry on the whole contributes about 5% to global anthropogenic CO_2 emissions, making the industry an important sector for CO_2-emission mitigation strategies. CO_2 is emitted from the calcination process of limestone, combustion of fuels in the kiln, as well as from power generation. The average intensity of carbon dioxide emissions from total global cement production amounts to 222 kg of C/t of cement (Worrell et al., 2001). Globally, 3.5 billon tonnes of cement is produced annually. Major countries in worldwide cement production from 2011-2016 shows China and India are top cement users globally (www.statista.com/).

The cement industry faces challenges including depleting raw material, fuel reserve and maintenance. Construction material such as concrete and stone are subjected to many weathering actions of physical and chemical factors (Saiz-Jimenez, 1997; LeMetayer-Levrel et al., 1999; Warscheid and Braams, 2000). Impermeability of concrete is considered as the first line of defense against any of the physiochemical deterioration process (Mehta et al., 1994). Durability of concrete is defined as its ability to resist chemical and physical attacks that lead to deterioration of concrete during its service life (Erdogan et al., 2002). These attacks include leaching, sulphate attack, acid attack, carbonation, alkali-aggregate reaction, freezing–thawing and abrasion. Durability of concrete is as important as its compressive strength. The environmental conditions such as humidity, temperature, water ingress and the mechanism of chemical transports are the major factors causing chemical and physical attacks (Karihaloo et al., 2002). The ingress of aggressive agents depends on various moisture transport processes, such as absorption, diffusion and permeability, and one or more of the deterioration mechanisms are influenced by the transport of these deleterious agents through concrete (Fig. 2.2).

At this stage, permeability influences the primary method of transport of moisture and aggressive ions into the concrete that is responsible for the increased rate of damage. The morphological changes in agents accelerate the penetration of these aggressive substances into the concrete, which in turn activates number of other mechanisms of deterioration.

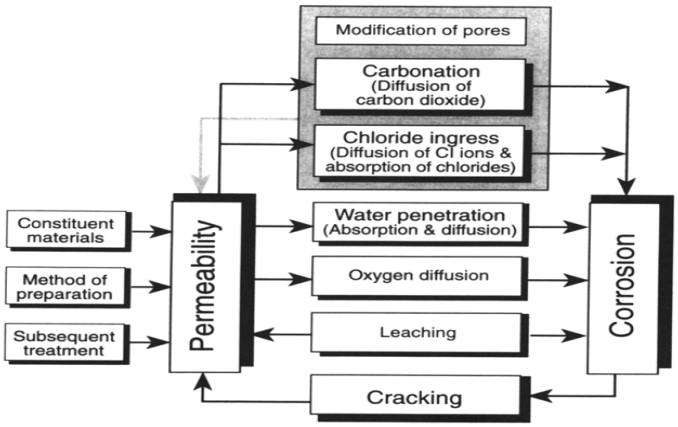


Fig. 2.2. Dependence of corrosion on permeation properties (Basheer et al.,1995).

2.2. Sustainable recourse utilization

Due to large-scale construction, natural sand is getting depleted at a very fast pace. Hence it makes necessary to find out an alternative to natural sand, which can be used as partial replacement of natural sand (fine aggregate) in cementitious matrix. The beneficial use of by-products in construction materials not only results in reducing the cost of construction materials but also helps in reducing disposal problem. The amount and type of waste materials are increasing because of the growing population. Many non-degradable materials remain present in environment for hundreds and thousands of years. These waste materials can cause disposal crises, thereby contributes to the environmental problems. The use of waste materials not only saves natural resources and dumping spaces but also maintains a clean environment. Partial replacement of aggregate in concrete with waste material is done to achieve the desired properties of concrete such as strength, durability and workability (Vasoya et al., 2015).

2.2.1. Utilization of waste in building materials

Human activities on earth produce considerable quantities of wastes amounting to more than 2,500 million tons per year, including industrial and agricultural wastes from rural and urban societies. This creates alarming problems to the environment, health and also the land filling. In todays scenario the concrete is most used manmade material in the world. Due to privatization and globalization, the construction of important infrastructure

projects like highways, airports, nuclear plants, bridges, dams etc. in India is increasing year after year (Vasoya et al., 2015).

The Indian construction industry alone consumes approximately 400 million tons of concrete annually and a relative amount of mortar too. Therefore, the demand of the required raw materials for construction is very high. This leads to not only faster depletion of natural resources but also increase the cost of construction of structures. To avoid the problems like cost hike and environmental impacts for mining the raw material, the alternate material or the partial replacements for the cement and aggregate should be developed by recycling of waste materials (Agrawal et al., 2014), which provides us the low cost and eco-friendly construction products. Use of the waste materials not only reduces the problem of landfilling, but also environmental and health concerns.

In view of this, people have been started searching for suitable other viable alternative materials which could be used either as an admixture or as a partial replacement to the conventional ingredients of concrete so that the existing natural resources could be saved to the possible extent, and could be made available for the future generation. In this process, different industrial waste materials such as quarry dust, tile waste, brick bats, broken glass waste, waste aggregate from demolition of structures, ceramic tiles, fly ash, blast furnace slag, electronic waste of discarded old computers, TVs, refrigerators, radios, waste paper mill pulp, iron filling, waste coconut shell, rice husk ash, marble dust powder, hypo sludge, machine crushed animal bones, chicken feather, eggs shell, granite quarry sludge, palm oil fuel ash, copper dust, human hair etc. have been tried as a viable substitute material to the conventional materials in concrete (Vasoya et al., 2015).

Several researchers have investigated the utilization of waste materials and industrial by-products in construction materials such as fly ash (Shi and Qian, 2003); crushed stone dust (Sahu et al., 2003); sewage sludge ash (Cyr et al., 2007); silica fume (Scott and Singh, 2011) and waste foundry sand (Santurde et al., 2011).

The need for utilization of wastes as construction material arises due to the following reasons:

- High energy cost in production of cement and aggregates
- Solve environmental problems caused by the generation of wastes and dumping
- Securing ample supply for replacement of concrete aggregates to the construction industry
- Prolong durability of concrete

Charkradhara et al. (2011) investigated the influence of different amounts of recycled coarse aggregates obtained from a demolished 15 years old culvert in recycled aggregate concrete (RAC). Due to high absorption capacity of old mortar adhered to recycled aggregates, water absorption of the recycled aggregate concrete were 2.61 and 1.82% higher than those of normal concrete. Khatib (2005) also investigated the properties of concrete containing fine recycled aggregate (crushed concrete (CC) or crushed brick (CB)). The fine aggregate in concrete was replaced with 0%, 25%, 50% and 100% CB or CC. Results showed that concrete including up to 50% CB displayed similar long-term strength to that of the control. Even at 100% replacement of fine aggregate with CB, the reduction in strength was only 10%. Rakshvir and Barai (2006) studied various mechanical properties of recycled concrete aggregates. The percentages of recycled concrete aggregates were varied and it was reported that properties such as compressive strength showed a decrease of up to 10% as the percentage of recycled concrete aggregates increased. However, water absorption of recycled aggregates was found to be greater than natural aggregates.

Ghernouti et al. (2011) investigated the use of Plastic Bag Waste as substitution of a variable percentage of sand such as 10, 20, 30 and 40 %. They found that bulk density decreased considerably for all concretes with the content of replacement of sand by plastic waste that also becomes lighter with 40% of plastic waste. They also reported that a fall in compressive strength at 28 days about 10 and 24 % containing 10 and 20 % of waste respectively.

Kanagalakshmi et al. (2015) observed the potential use of both agricultural waste namely rice husk ash (RHA) and industrial waste copper dust (CD) as raw material in production of concrete. It was concluded that concrete with 20% RHA and 40% CD had shown high compressive strength. Pande and Makarande (2013) concluded that rice husk ash (RHA), which is the waste product of agricultural industry, could be used as materials in concrete by replacement of 12.5 %.

Prasanna and Rao (2014) carried out a study by utilizing E- waste particles as coarse aggregates in concrete with percentage replacement from 0% to 20% i.e. (5%, 10%, 15% and 20%). Their results concluded that the compressive strength of concrete was found to be optimum when coarse aggregate was replaced by 15% E-Waste whereas beyond it, the compressive strength decreased. Cwirzen (2010) studied the effect of nano-materials on physical properties of cementitious matrix. The compressive strength increased up to 50% by addition of 0.23 wt% of carbon nano-tubes. Alzaed (2014) observed that iron filings, which are very small pieces of iron that look like a light powder can be used in concrete mix with an effective compressive strength increase by 17% when 30% of iron filling was added.

Ammash et al. (2009) and Ahmad et al. (2013) studied the possibilities of substituting Waste Glass as a fine aggregate in concrete. The waste glass was used as partial weight replacement of sand with percentages of 10, 20, 30 and 40 %. The former reported that waste glass aggregate could be satisfactorily substituted for natural fine aggregate at replacement levels up to 20%. Ahmad et al. (2013) reported that 20% replacement of fine aggregates by waste glass showed 25% increase in compressive strength and replacement by waste glass up to 30% by weight showed 9.8% increase in compressive strength at 28 days.

Maslehuddin et al. (2003) presented a comparative study about the use of steel slag aggregate concrete and crushed limestone concrete. It was concluded that the compressive strength of steel slag aggregate concrete was marginally better than that of crushed limestone aggregate concrete. Alizadeh et al. (2003) concluded that utilization of steel slag as aggregate in concrete has advantages compared to natural aggregate. Also,

results of the experiments that are carried out on hardened concrete indicated that slag aggregate concretes achieved higher values of compressive strength. Shekarchi et al. (2004) conducted comprehensive researches on the utilization of steel slag as aggregate in concrete and reported that utilization of steel slag as aggregate is advantages when compared with normal aggregate mixes. Manso et al. (2004, 2006) presented a study in which better quality concrete was obtained by electric arc furnace slag. It was concluded that arc furnace slag enhanced concrete properties.

2.2.2 Steel Slag

One of the fastest extending sectors of the manufacturing industry all over the world is stainless steel (Huaiwei et al., 2011). Steel slag is generated as byproduct or waste material from the steel industries. About 2–4 ton of wastes are being generated per ton of steel during the steel production from steel industries. The main constituents in steel slag are lime, silica and iron oxide and traces of alumina, magnesia, sulfur, and oxides of manganese and titanium can also be found (Tarco, 2000). Its chemical composition consists of majorly CaO 45–60%, SiO_2 10–15%, Al_2O_3 1–5%, Fe_2O_3 3–9%, FeO 7–20% and MgO 3–13% .The presence of compounds like C_3S, C_2S, and C_2F endorses steel slag cementitious properties. Its characteristics properties are wear-resistant, hard, adhesive, rough, porous and highly alkaline (Das et al., 2007; Devi et al., 2014). Simple disposals of these wastes in landfills are becoming less attractive since it not only occupies plenty of land but also increases the disposal costs and a considerable amount of steel slag produced is discarded (Kang et al., 2004). It is estimated that over 50% of such waste is currently discarded and as it becomes more difficult to secure areas for landfill, effective technologies for the reuse of them are highly desired.

Natural weathering proceeds too slowly and technique like autoclaving consume high energy. Hence more sustainable bio geochemical inspired methods need to be considered to treat and replace the aggregates with slag in concrete. Steel slag can be used as a cementing component in several cementing systems. The presence of free lime particles can lead to volume expansion resulting in cracking of aggregate in concrete (Tarco, 2000). Some material being able to consume free lime and to eliminate potential expansion must be used together with steel slag (Shi 2004). Along with the replacement,

durability enhancement of the concrete is considered to improve the concrete strength and life span. The excessive free lime content can be utilized as the source of calcium for calcium carbonate precipitation in presence of suitable microorganism.

There are three types of steel industry slag, each named after the process from which it is produced: Blast Furnace (BF) iron slag, Basic Oxygen Furnace (BOF) steel slag, and Electric Arc Furnace (EAF) steel slag. Blast Furnace slags are used for iron production, while EAF slag and BOF slag are used for steel production. While most of the blast furnace slag is reused, a huge amount of steel slag produced is discarded (Kang et al., 2004). All three types of slag are primarily comprised of fluxing agents (mainly lime) used during the iron and steel making process and the molten impurities of iron or steel. The composition of blast furnace is primarily silica and alumina from the original iron ore, with calcium and magnesium oxides from the added flux. Fluxing agents are also added in the BOF and EAF processes; hence the chemical composition of slag from these processes is similar to BF slag. However, the iron and manganese contents in BOF and EAF slags are substantially higher (Proctor et al., 2000).

The utilization of steel slag as asphaltic concrete aggregate finds its applications in different sectors including use as an, road base, airport runways, soil stabilizer and railroad ballast. Heaton and Bullen (1982) reported that specific slag mixtures comprising air-cooled slag, granulated slag and BOS steel slag in the proportion 50:20:30 exhibited properties adequate for consideration as bound base materials in concrete. In Batlimore (Graham, 1992) the exceptional characteristics demonstrating one of the benefits of using slag bituminous concrete is high skid resistance (under wet or dry conditions) provided throughout the service-life of the pavements. Another study (Stock et al., 1996) reported the use of steel slag in bituminous road construction in South Yorkshire and surrounding region for the past 60 years.

2.3 Biomineralization in enhancing durability of building materials

Biomineralization has become important research topic during the last decade to enhance durability of cement using calcifying bacteria and develop sustainable cementitious building material. Biomineralization of calcium carbonates, that is the biologically

controlled or mediated/induced mineralization of $CaCO_3$ which is an ubiquitous process resulting in bio minerals produced by a range of taxa, from bacteria and archaea to eukarya (Lowenstam and Weiner, 1989; Mann, 2001; Gower, 2008; Meldrum and Colfen, 2008; Dittrich and Sibler, 2010).

In case of biologically controlled mineralization (BCM), minerals are synthesized directly at a specific location within or on the cell and only under particular conditions. In most cases, BCM happens intracellularly, where the lipids, proteins and polysaccharides make a stable matrix for the cations to condense, and for the minerals to grow in a constrained space. Examples of controlled mineralization include magnetite formation in magnetotactic bacteria (Bazylinski et al., 2004) and silica deposition in unicellular algae coccolithophores and diatoms (Barabesi et al., 2007). Minerals that form by biologically induced mineralization (BIM) processes generally nucleate and grow extracellularly as a result of metabolic activity of the organism and subsequent chemical reactions which involve metabolic byproducts. Bacterial cell walls or polymeric materials (exopolymers) exuded by bacteria including slimes, sheaths, or biofilms, and even dormant spores can act as important sites for adsorption of ions, mineral nucleation and growth (Banfield and Zhang, 2001; Bäuerlein, 2003). Knorre and Krumbein (2000) concluded Microbes induce calcium carbonate precipitation (MICCP) as a byproduct of microbial metabolic processes.

Microbially induced calcium carbonate precipitation, part of biomineralization has been the primary focus of research in bio geotechnical engineering too. The carbonate precipitation induced by microbial metabolic activities is advantageous as it is pollution free, natural and a continuous process. The promising results of this technology invited researchers from worldwide to harness these carbonates precipitation for various purposes.

2.3.1. Microbially induced calcium carbonate precipitation (MICCP)

MICCP has attained immense interest in the last 20 years and is found to be the primary focus of research in bio geo civil engineering because of its numerous applications. The application of MICCP in remediation of building materials has brought another crucial

revolution in which applied microbiology and biotechnology have proved their potential for serving the problems of civil engineers. The use of bacteria for remediating building materials seems like a new idea but this conservation method mimics what nature has been doing for eternity. This technology has offered the benefits of being unique, self-healing and eco-friendly.

Mainly four groups of microorganisms are seen to be involved in this process:

- Photosynthetic organisms - such as cyanobacteria and algae
- Sulphate reducing bacteria- that are responsible for dissimilatory reduction of sulphates
- Organisms utilizing organic acids
- Organisms that are involved in nitrogen cycle (by ammonification of amino acids /nitrate reduction/ hydrolysis of urea) (Stock Fischer et al., 1999; Hammes and Verstraete, 2002; Jargeat et al., 2003).

The most common form of MICCP in aquatic environments is caused by photosynthetic organisms such as cyanobacteria and algae (McConnaughey and Whelan, 1997).

Heterotrophic organisms can also precipitate calcium carbonates, by the production of carbonate or bicarbonate and modification of the environment to favor precipitation (Castanier et al., 1999). The abiotic dissolution of gypsum ($CaSO_4.2H_2O$) provides an environment rich in both sulphate and calcium ions.

Third pathway includes bacteria using organic acids as their sole source of carbon and energy. Such acids include citrate, oxalate, acetate, glyoxylate, succinate and malate. The consumption of these acids results in pH increase, thereby leads to the precipitation in the presence of calcium ions (Knorre and Krumbein, 2000; Braissant et al., 2002).

The fourth pathway for MICCP is by organisms involved in the nitrogen cycle, via, nitrate reduction, ammonification of amino acids and the hydrolysis of urea. Hydrolysis of urea by enzyme urease is the simplest of all mechanisms described for this process.

Calcium carbonate is readily precipitated in the presence of calcium under these conditions as:

$CO(NH_2)_2 + 2H_2O \rightarrow CO_3^{2-} + 2NH_4^+$

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

2.3.2. Enzyme Urease

Urease is a nickel-containing metalloenzyme (Fig. 2.3.), which is found in a wide range of microorganisms and plants and some of these produce this enzyme in large quantities (Mobley et al., 1989). The commercial demand for urease is not exceptionally high and urease is available in industrial quantities from Roche for use in the diagnostic and high technology specialist ceramics fields (Gauckler and Baader, 1999; Roche, 2001). It is thus expensive and is of a higher purity than what is required for applications in biocementation.

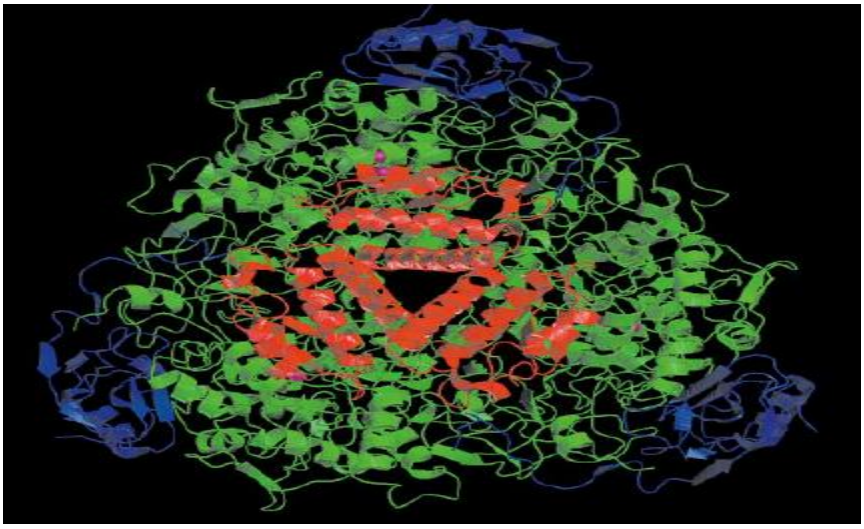


Fig. 2.3. Urease from Bacillus pasteurii. The green, blue and red ribbons represent α, β and γ subunits while magenta spheres are nickel ions (Benini et al., 1999).

Organisms with urealytic ability mostly use urea as a source of nitrogen and transport urea actively or by passive diffusion into the cell cytoplasm, where urease hydrolyses urea releasing two ammonium molecules as the end product. These products can then be directly assimilated into biomass either via the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway or by the action of glutamate dehydrogenase (GDH) (Tyler, 1978).

The use of ureolytic bacteria in biotechnological applications is also appealing because of many reasons:

- Firstly the enzyme urease is widespread amongst bacteria.
- Another benefit of ureolytic bacteria is that urea, an important nitrogen compound found in natural environment, is a fairly inexpensive substrate (Hammes et al., 2003).
- Also, the use of bacteria to raise the pH in the environment is preferable to the direct injection of a base because the gradual hydrolysis of urea is likely to promote a wider spatial distribution of calcite, whereas the direct addition of base is likely to cause immediate precipitation at the injection site (Ferris et al., 2003).

For biocementation purposes, an ideal microbial source of urease should have the following properties:

i. High urease production capacity
ii. Non pathogenic
iii. Ability to produce urease in the presence of ammonium
iv. Consistent production (reliable)
v. High stability (robust)

2.3.3. Microbially induced calcium carbonate precipitation via urea hydrolysis

The precipitation of carbonates via urea hydrolysis by ureolytic bacteria is the most easily controlled mechanism of MICCP with potential to produce high amounts of carbonates in short period of time (Fig. 2.4). During microbial urease activity, 1 mole of urea is hydrolyzed intracellularly to 1 mole of ammonia and 1 mole of carbonate. This spontaneously hydrolyzes to form additional 1 mole of ammonia and carbonic acid as follows (Hammes and Verstraete, 2002):

$$CO(NH_2)_2 + H_2O \rightarrow NH_2COOH + NH_3$$

$$NH_2COOH + H_2O \rightarrow NH_3 + H_2CO_3$$

These products equilibrate in water to form bicarbonate, 1 mole of ammonium and hydroxide ions as end products, which give rise to pH.

$$H_2CO_3 \rightarrow HCO_3^- + H^+$$

$$2\,NH_3 + 2\,H_2O \rightarrow 2NH_4^+ + 2OH^-$$

$$HCO_3^- + H^+ + 2NH_4^+ + 2OH^- \rightarrow CO_3^{2-} + 2NH_4^+ + 2H_2O$$

$$Ca^{2+} + Cell \rightarrow Cell\text{-}Ca^{2+}$$

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

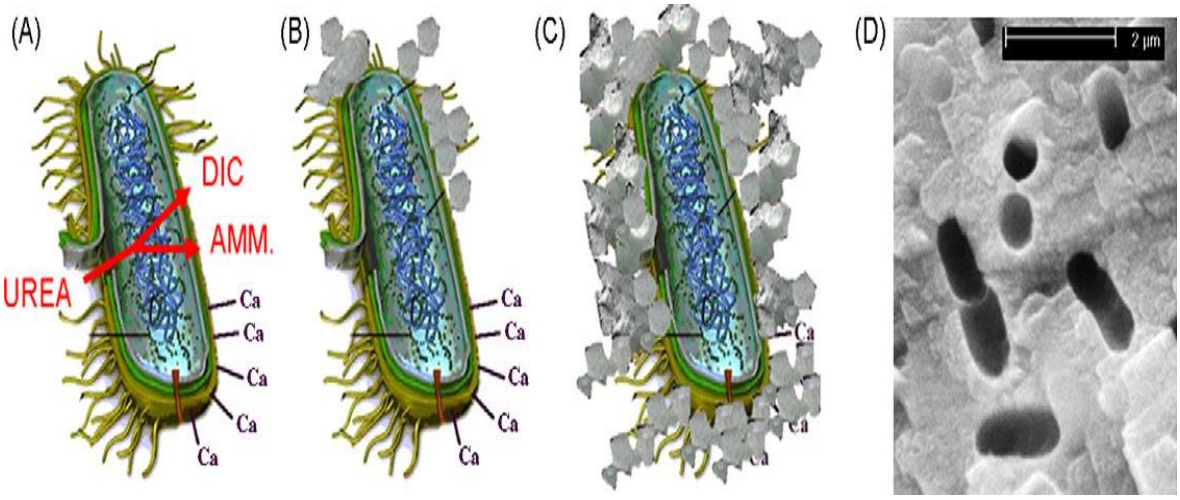


Fig. 2.4. Simplified representation of the events occurring during MICCP Calcium ions in the solution are attracted to the bacterial cell wall due to the presence of negative charge. Upon addition of urea to the bacteria, dissolved inorganic carbon (DIC) and ammonium (AMM) are released in the microenvironment of the bacteria (A). In the presence of calcium ions, this can result in a local super saturation, which leads to heterogeneous precipitation of calcium carbonate on the bacterial cell wall (B). After a while, the whole cell becomes encapsulated by the precipitation (C), limiting nutrient transfer, resulting in cell death. Image (D) shows the imprints of bacterial cells, which are involved in precipitation (Source: Hammes and Verstraete 2002).

Bacterial surfaces also play a vital role in calcium precipitation (Fortin et al., 1997). Due to the presence of several negatively charged groups, at a neutral pH heterogeneous nucleation is favored as positively charged metal ions bound on bacterial surfaces (Douglas, 1998; Bauerlein, 2003). Ferris and Stehmeier (1992) suggested that there is considerable evidence that microorganisms might act as crystal nucleating agents during

mineral precipitation. As bacteria have high surface to volume ratio, they prove to be ideal crystal nucleation sites (Warren and Haack, 2001).

Gram-negative bacteria's cell membranes are dominated by presence of carboxylate groups, as well as phosphate moieties resulting in net negative charge (Schultze-Lam et al., 1996) while gram-positive bacteria have lipopolysaccharide (LPS) and peptidoglycan having net negative charge (Fig. 2.5). The overall anionic nature of bacterial cells in most aquatic environments is result of pKa values exhibited by carboxyl and phosphoryl groups (Ferris and Beveridge, 1985; Warren and Haack, 2001). Under normal conditions, divalent cations like calcium and magnesium contribute to the stability of techoic and teichuronic acid polymers, lipopolysaccharides (LPS) and S-layers. This reaction of binding of divalent cations to the cell wall forms the beginning of crystallization (Southam, 2000). Not only does the cell surface features of bacteria contribute to crystal nucleation site formation but also influence cellular processes on immediate surroundings of the bacteria (Schultze-Lam et al., 1996). Microorganisms are surrounded by thin watery layer, which physically creates microenvironment around them (Schultze-Lam et al., 1996; Warren and Haack, 2001). This layer forms the interface between microbial cell and outside environment, in which the chemical species different to those of outside environment can prevail.

Typically, carbonate precipitates develop on the external surface of bacterial cells by successive stratification (Pentecost and Bauld, 1988; Castanier et al., 1999) and bacteria eventually can be embedded in growing carbonate crystals (Rivadeneyra et al., 1998; Castanier et al., 1999). Probable biochemical reactions in urea-$CaCl_2$ medium to precipitate $CaCO_3$ at the cell surface can be summarized as follows:

$$Ca^{2+} + Cell \rightarrow Cell\text{-}Ca^{2+}$$

$$Cl^{-} + HCO^{3-} + NH_3 \rightarrow NH_4Cl + CO_3^{2-}$$

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

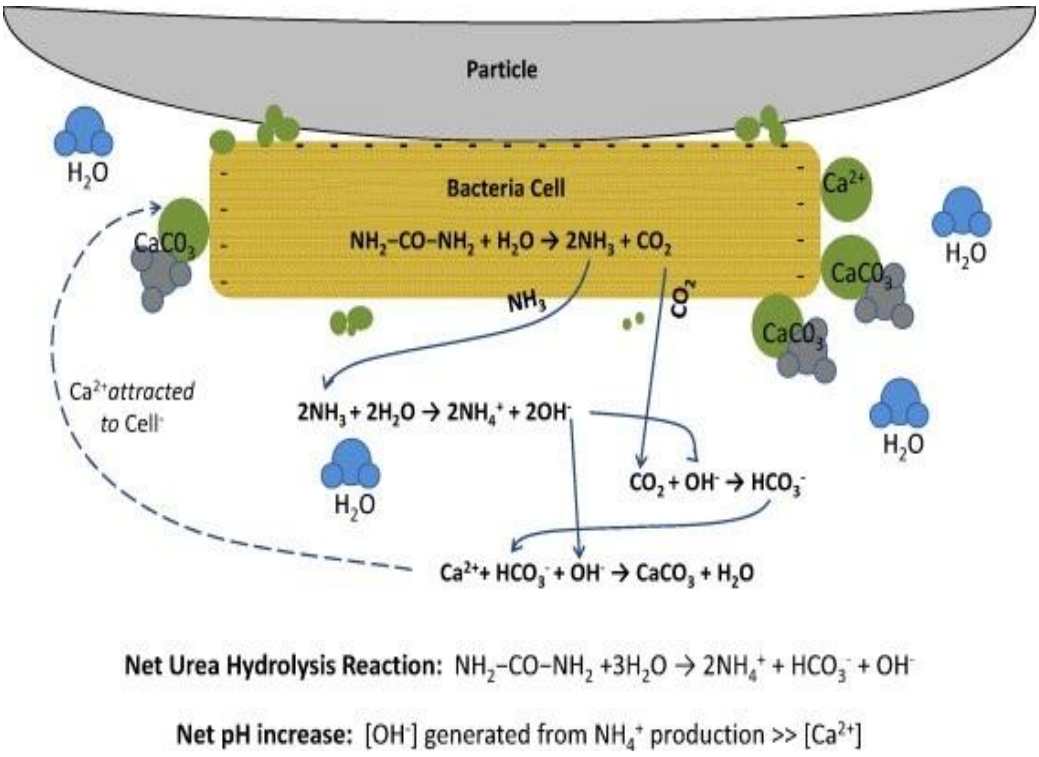


Fig. 2.5. Bacteria serving as nucleation site for $CaCO_3$ precipitation in the sand particle. (Source: DeJong et al., 2010)

Several authors have investigated various conditions for calcium carbonate precipitation via Urea hydrolysis however the actual role of the bacteria in the precipitation remains a matter of debate. Some authors believe this precipitation to be an accidental by-product of the metabolism (Knorre and Krumbein, 2000) while others think that it is a specific process, which has ecological benefits for the precipitating organisms (Ehrlich, 1996; Mc Connaughey and Whelan, 1997).

In a nutshell, the key factors that MICCP process depends on are: (1) Urease, (2) Calcium concentration, (3) pH of the surrounding solution, (4) Availability of nucleation sites, (5) Saturation index, (6) Bacterial species (Achal et al., 2015).

CHAPTER 3

MATERIAL AND METHODS

3.1. Isolation and identification of calcifying bacteria

3.1.1. Sample collection

Steel Slag is the waste generated from steel industries. Steel slag samples were received from Tata Steel Industries, Jamshedpur, India. These samples were passed through pre-determined sieve size of 4.75 mm and were collected to execute further experimental work.

3.1.2. Isolation and enrichment of ureolytic bacterial species

For the isolation and enrichment of ureolytic bacteria, two-gram of respective steel slag sample was added in 100 mL nutrient broth (Hi-Media, Mumbai, India) (pH 8.0) containing 2% urea and incubated at 37°C for 120 hrs at 120 rpm. For isolation and enumeration of cultivable bacteria, the sample was serially diluted in saline (0.85%) and plated on nutrient agar medium. The plates were then incubated at 37°C overnight. The grown bacterial colonies from the master plates were sub cultured several times on nutrient agar plates. Well-segregated bacterial colonies were then picked and further selected for ureolytic activity on Urea Agar Base medium (HiMedia, India) for preliminary estimation of urease activity. Intensity of pink colored halo zone around colonies indicated urease enzyme production thus qualitatively confirming ureolytic activity (Fig 3.1). These colonies were selected for quantitative estimation of Urease.

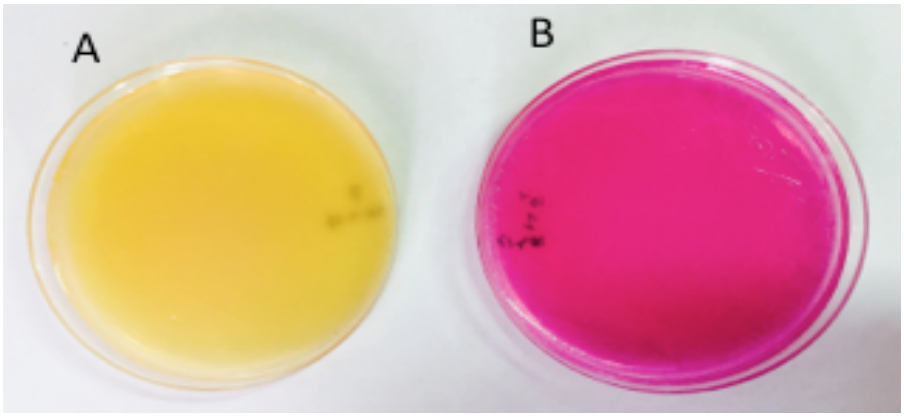


Fig. 3.1. Enrichment of ureolytic isolates on urea agar medium: A) Negative control B) Positive control.

3.1.3. Urease Assay

For estimation of urease activity in urease positive isolates, inoculum size of 1% (OD_{600} 0.5) of each isolate were introduced into 250 ml flasks containing 100 ml Nutrient broth supplemented with 2% urea (pH 8·0) and incubated under shaking conditions at 37°C (120 rpm). One ml of overnight grown culture from each flask was centrifuged (8000 rpm for 5 mins) and the supernatant was collected. The amount of ammonia released from urea in the medium was then determined according to the phenol-hypochlorite assay stated by Natarajan (1995). The supernatant (250 ml) was added to solution containing 1 ml of 0.1 M potassium phosphate buffer (pH 8.0) and 2.5 ml of urea (0.1 M). The solution was incubated for 5 minutes at 37°C followed by addition of phenol nitroprusside and alkaline hypochlorite, 1 ml each. The assay mixture was then incubated for 25 minutes at 37°C gives blue colour as shown in Fig. 3.2. Optical density was measured at 626 nm. Ammonium chloride (50- 1000 µM) was used as the standard for estimation. One unit of urease can be defined as the amount of enzyme hydrolyzing one µ mole urea per minute.

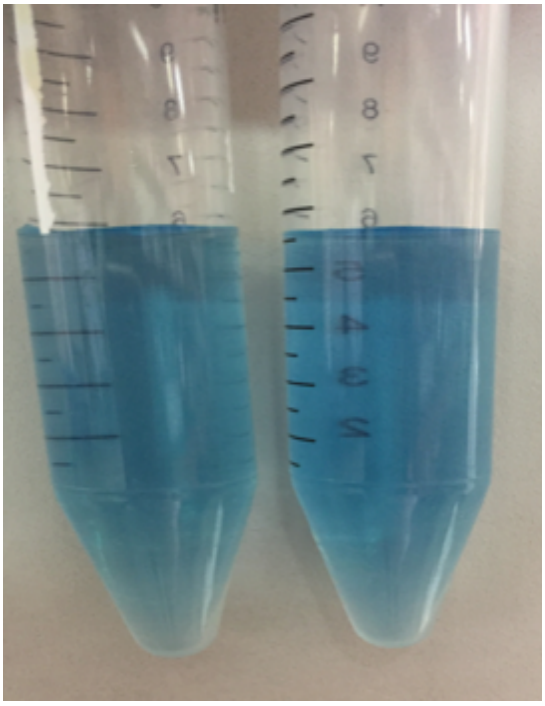


Fig. 3.2. End point of urease assay estimation

3.1.4. Molecular characterization

3.1.4.1. Isolation of genomic DNA

- A single colony of bacterial isolate was inoculated into 25 ml of nutrient broth in a 250 ml flask and was incubated for 14-18 hours at 37°C under shaking condition (120 rpm).
- Liquid culture (2.0 ml) was harvested by centrifugation (Eppendrof microfuge) at 8,000 rpm for 1 min.
- The cell pellet was resuspended in 800-µl saline-EDTA and approximately 10µg crystalline lysozyme was added.
- The cell suspension was mixed thoroughly by inverting the Eppendrof tube several times and incubated for 30 mins at 37°C.
- After addition of 200 µl SDS (10%), the cell suspension was incubated again at 65°C for 15 min.
- The cell suspension was extracted with organic solvents to remove proteins and cell debris: first, with an equal volume phenol:chloroform:isoamyl alcohol (25:24:1) solution, and centrifuged 10 min at 12,000 rpm.
- The upper aqueous phase was then extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1).
- To precipitate extracted nucleic acids, 0.7-volume isopropanol was added to the aqueous phase followed by 10 min centrifugation at 12,000 rpm.
- The DNA pellet was washed with 750 µl EtOH (70%) and microfuged another 10 min.
- Finally, the pellets were resuspended in 40 µl TE buffer/milliQ water and stored at 4°C for further use.

3.1.4.2. Electrophoresis of DNA on agarose gels

- DNA was loaded on agarose gels (0.8 % w/v) prepared in 1X TBE, pH 8.0 using a 6X loading dye (appendix I).
- Ethidium bromide (0.5 µg/ml) was added prior to pouring to stain the gel.
- The nucleic acids were then electrophoresed at 70 volts for 45-60 minutes and visualized on a U.V. transilluminator.

3.1.4.3. Spectrophotometric quantification of DNA

The concentration of the extracted DNA in suspension was estimated by spectrophotometric measurement at A_{260}. For double-stranded DNA suspensions, an OD of 0.5 at a wavelength of 260 nm, using a cuvette with 1 cm light path is equal to a concentration of 50 μg/ml. The quality of the DNA was evaluated by measurement of the A_{260}/A_{280} and the A_{230}/A_{260} ratios. Ideally, the A_{260}/A_{280} ratio should be 1.8-2.0 while the A_{230}/A_{260} ratio should be 0.3- 0.9. Ratios (A_{260}/A_{280}) less than 1.8 indicate protein or phenol contamination, while ratio value greater than 2.0 indicate the presence of RNA.

3.1.4.4. Amplification of 16S rDNA

DNA amplification was performed with Genamp 2700 PCR system (Applied Biosystem, USA). The polymerase chain reaction (PCR) provides a rapid and highly sensitive method for the primer-mediated enzymatic amplification of specific target sequences in genomic DNA resulting in the exponential increase of target DNA copies. For amplification of 16S rRNA gene the following primers were used: Forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 5'-ACGGGCGGTGTGTTC-3'.The amplification of 16S rDNA from isolates was carried out with a GenAmp thermocycler (Applied Biosystem, USA). PCR conditions were as follows: Preheating at 92°C for 120 s followed by 36 cycles of 92°C for 60 s, 48°C 30 s and 72°C for 120 s and final extension 72°C for 370 s. Successful amplifications were confirmed by agarose gel (0.8% w/v) electrophoresis and ethidium bromide staining.

3.1.4.5. Restriction fragment length Polymorphism (RFLP) analysis

Restriction fragment length Polymorphism (RFLP) analysis of 16S rDNA PCR products was carried out by using restriction enzymes (Thermo Scientific) as mentioned in the Table 3.1.

Table 3.1. Restriction enzymes used in RFLP analysis

Restriction enzyme	**Restriction site**	**Digestion condition**	**Thermal Inactivation**
Alu1	5'..A G↓C T..3' 3'..T C↑G A..5'	37°C for 1-16 hrs	65°C for 20 mins
Hinf1	5'..G↓A N T C..3' 3'..C T N A↑G..5'	37°C for 1-16 hrs	65°C for 20 mins
Taq1	5'..T↓C G A..3' 3'..A G C↑T..5'	65°C for 1-16 hrs	Inactivated by EDTA

Mixture for the restriction reaction of volume 20 µl was prepared as follows

Enzyme 1 µl
Buffer 2 µl
DNA 5 µl
Water 12 µl

Restriction enzyme digested fragments were visualized on 2% of ethidium bromide stained agarose gel after electrophoresis at 70 volts for 45-60 minutes using a U.V. transilluminator.

3.1.4.6. Ligation of 16S rDNA in pMDT20 vector

The 16S rDNA PCR products were cloned using the restriction independent Insta Cloning Kit, following the manufacturer's protocol (Fermentas, USA). The 16S rDNA amplicon was ligated into pMDT20 vector. The reaction mixture was prepared as described below and incubated overnight at 4°C.

PCR product 3 µl
Vector 1 µl
Ligase Mixture 5 µl

3.1.4.7. Genetic Transformation using $CaCl_2$

- A single colony of E. coli DH5α from a freshly grown plate was inoculated into 25 ml of LB broth in a 250 ml flask and incubated the culture for 16-20 hrs at

37°C under shaking condition (120 rpm).

- Aseptically 200 ml of the above-saturated culture was transferred into 25 ml of fresh LB broth in a 250 ml flask.
- The culture was further incubated with vigorous shaking at 37°C for 2-3 hrs.
- To monitor the growth of the culture, the OD_{600} was determined at every one-hour (OD_{600} should be ~ 0.5).
- The above culture was transferred to sterile disposable, ice-cold 50 ml polypropylene tubes.
- The culture was cooled to 0°C by storing the tubes on ice for 10 min.
- The cells were harvested by centrifugation at 8,000 rpm for 10 minutes at 4°C.
- The pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and stored on ice for 15 min.
- The cells were further recovered by centrifugation at 8,000 rpm for 10 min at 4°C.
- The cell pellet was resuspended in 1 ml of ice-cold 0.1 M $CaCl_2$.
- The plasmid DNA sample (~100 ng in a volume of 5 ml or less) was added to each tube.
- The content of the tubes were mixed gently and stored the tubes on ice for 30 minutes.
- The tubes were incubated in a circulating water bath that has been preheated to 42°C for exactly 2 minutes without shaking.
- The tubes were rapidly transferred to an ice bath and chill the cells for 1-2 minutes.
- One ml of LB broth was added to each tube and incubated the cultures for 45-60 minutes at 37°C to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid.
- One hundred micro liters of transformed cells was spread on each 90 mm Luria agar-Ampicillin-X-Gal-IPTG plates and incubated at 37°C. Transformed colonies should appear in 12-16 hours.

3.1.4.8. Blue/white screening for recombinant plasmids

- After transformation of the ligated product, the E. coli DH5α (Lac Z^-) bacterial host cells were plated on Luria agar medium for selection of transformants containing 50-µg/ml ampicillin.
- X-Gal and IPTG were used to screen for colonies containing a recombinant plasmid.
- The cloning site in the pMDT20 vector is located in the multiple cloning site (mcs) of the plasmid's lacZα gene; if no insert is present, functional β-galactosidase is produced, and the transformed bacterial colony is blue. These few blue colonies occur due to the presence of supercoiled vector molecules, which have escaped linearization.
- However, if the host cell receives a recombinant plasmid containing a 16S rDNA insert in the lacZα gene, the resulting transformants colony is white (Lac Z^-).

3.1.4.9 Bacterial Plasmid isolation

Plasmid isolation was carried out with the aid of QIAprep® Spin Miniprep Kit.

1. 1-5 ml bacterial overnight culture, centrifuged at >8000 rpm for 3minutes at room temperature (15-25˚C).
2. In 250µl of Buffer P1 (Appendix II) pellet obtained was resuspended and transferred it into microcentrifuge tube.
3. 250µl of Buffer P2 (Appendix II) and mix thoroughly by inverting microcentrifuge 4-6 times, until the solution becomes clear.
4. 350µl Buffer N3 (Appendix II) and immediately mixed it by inverting the tube.
5. The solution was centrifuged at 13,000 rpm for 10 minutes in a tabletop microcentrifuge.
6. 800 µl of supernatant from step 5 added into the QIAprep 2.0 spin column and centrifuged the solution at 13,000 rpm for 30-60 seconds and discard the flow-through.
7. 0.5 ml of Buffer PB (Appendix II) was added to carry out washing in QIAprep 2.0 spin column.

8. 0.75 ml Buffer PE (Appendix II) was used to wash again, centrifuged for 30-60 seconds and discarded the flow-through. QIAprep 2.0 spin column was transferred to the collection tube.
9. Centrifuged for 60 seconds to remove residual wash buffer.
10. QIAprep 2.0 column was placed in a clean 1.5 ml microcentrifuge tube, added 50 µl Buffer EB to elute DNA. Let column stand for 60 seconds and centrifuge for 60 seconds.

3.1.4.10. Colony PCR of bacteria

1. In 5 µl of ultrapure water bacterial cells were suspended and incubated at 95ºC for 10 minutes.
2. The tubes were then quick chilled on ice and vortex for 5 seconds then 3 µl of supernatant was transferred in fresh tube containing 17 µl of PCR master mix

PCR Reaction mixture

Components	Concentration
MQ Water	11.2 µl
10X Buffer	2 µl
dNTPs	1.5 µl
M13F	1 µl
M13R	1 µl
Taq polymerase	0.3 µl

3. The PCR amplification was carried out with M13-forward/M13-reverse plasmid primers. The PCR program for amplification was carried out as following:

PCR Programme

Stage	Temperature	Time	
Initial denaturation	94˚C	5 minutes	
Final denaturation	94˚C	30 seconds	35 cycles
Annealing temp	55˚C	1 minute	
Initial extraction	72˚C	1 minute	
Final extraction	72˚C	10 minutes	
Extending	4˚C	∞	

4. Amplified products were visualized on 0.8% (w/v) agarose gel.

3.1.4.11. Sequencing

The 16S rDNA inserts were sequenced for both strands using M13 forward and reverse primers, used for pMDT20 vector. The sequence was generated by chain termination method using an Applied Biosystems automatic sequencer (DNA Sequencing Facility, Department of Biochemistry, South Campus, Delhi University, New Delhi, India).

3.1.4.12. Analysis of sequence data

Using EZ Biocloud big data analysis platform, sequence homology search was carried out. Homologous sequences were retrieved and aligned using Clustal W (Thompson et al., 1994). Evolutionary distances of aligned sequences were calculated using Kimura 2 parameter. Phylogenetic tree was constructed using neighbour joining method of MEGA 6 package (Tamura et al., 2013).

3.1.5 Morphological and physiological characterization of selected bacterial isolate

The characterization of the selected bacterial isolate was done by conventional physiological and biochemical tests described in Bergey's Manual of Systematic Bacteriology.

3.1.5.1. Growth kinetics of bacterial isolates

The growth profile of bacterial isolate in media was tested by measuring the absorbance (OD_{600}) at regular time intervals of 2 hrs for a total of 30 hrs and growth curve was plotted after incubation at $37^{o}C$.

3.1.5.2. Gram staining

Bacterial smear was prepared from actively growing cells and spread on a glass slide, fixed by heating. Gram staining was performed using HiMedia Gram stains-Kit. Crystal violet was flooded for 1 minute. It was followed by brief washing in water till excess crystal violet is removed. Gram iodine was flooded for 1 minute and washed with water. Further, it was decolorized with ethyl alcohol and washed immediately in tap water.

Safranin was used to counter stain for 30 sec and washed with water to remove the excess stain. The slides were visualized under microscope at different magnifications (10X, 40X, 100X).

3.1.5.3 Coagulase test

This was done to check the pathogenicity of the isolated bacteria. It was carried out using Hiaureus^{TM} Coagulase Confirmation kit (HiMedia) according to the manufacturer's instructions. Coagulase test is used to differentiate *Staphylococcus aureus* (positive) from Coagulase Negative Staphylococcus (CNS). Coagulase is an enzyme produced by *S. aureus* that converts (soluble) fibrinogen in plasma to (insoluble) fibrin, which results in the formation of a clot (www.microbeonline.com). Clot formation indicates the presence of *Staphylococcus aureus* whereas absence of clot indicates the absence of this organism, which further confirms the isolate as non-pathogenic.

3.1.5.4 Mannitol salt agar

Mannitol salt agar (MSA) was developed for the presumptive isolation of *S. aureus*, which is convenient for diagnostic laboratories. The efficiency of the tube coagulase test can be markedly improved by sequel testing of the isolate with Mannitol salt agar (Kateete et al., 2010). MSA is recommended for use as a selective and differential medium for the identification of pathogenic staphylococci. *Staphylococcus aureus* ferments mannitol and results in acid production as the result of fermentation of this sugar, which results in the formation of colonies with a yellow zone. To confirm fermentation of mannitol, growth of yellow colonies on MSA surrounded by yellow zones after 24 hours of incubation at 37°C indicated a positive result. Absence of these zones indicates a negative result and the isolate is non-pathogenic. Coagulase-negative strains are usually mannitol non-fermenters and therefore produce pink to red colonies surrounded by red-purple zones.

3.2 Study of properties at flask level

3.2.1 Precipitated carbonates measured by EDTA titration method

20 ml of aliquot was taken from the 250 ml flask. Final pH set at 12 of the test sample.

Pinch of Ammonium purpurate was added as an indicator and the mixture was finally titrated against 0.05 M EDTA. End point was noted from pink to purple (Fig. 3.3) which is easily visualized and the amount of soluble calcium (ppm) used was calculated by the volume of (EDTA used × 1000)/20

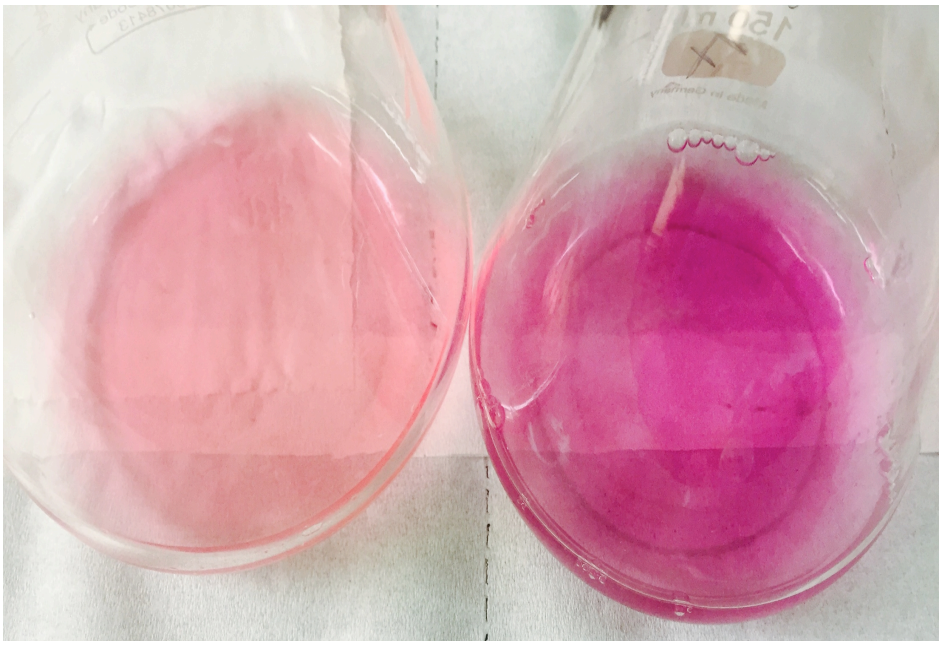


Fig. 3.3. End point from pink to purple in EDTA Titration method

3.2.2 Soluble Calcium Content Estimation in flask level

In order to check the efficacy of bacterial formulations, 1% inoculum was inoculated into 100 ml Nutrient broth supplemented with 2% urea and 25 mM $CaCl_2$ (NBUC) as described earlier. In another set, fresh bacterial cells (1%) were inoculated into NBU media along with 5gm Slag and 2% urea. Both sets were incubated at 37^{o}C in rotating shaker at 120 rpm for 72 hours for precipitation of $CaCO_3$ which is visible along the walls of flask (Fig. 3.4). The aliquots from flasks were taken at regular time intervals of 24 hours and the broth was centrifuged to quantify soluble Ca^{2+} in the supernatant by EDTA titration method as described before in 3.2.1.

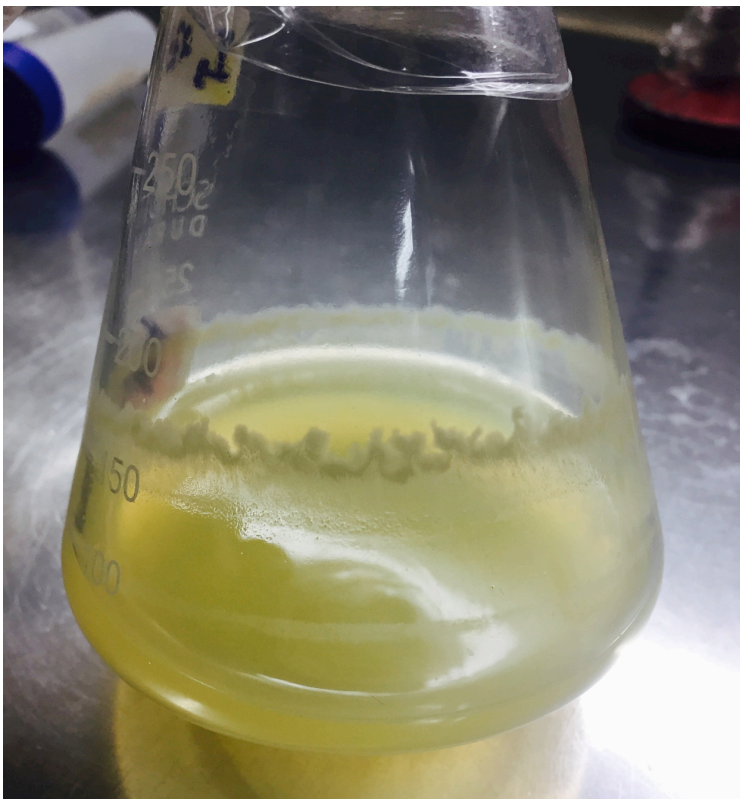


Fig. 3.4. Precipitates of $CaCO_3$ crystals along the walls as well as at bottom of flasks inoculated with PP9

3.2.3 Estimation of Changes in pH at flask level

To estimate the changes in pH, aliquot from flask was taken at regular interval of 24 hrs and the variations were recorded.

3.3 Study of Cement motar cubes properties

3.3.1 Preparation for Cement motar cubes

To study the effect of isolated bacteria on mechanical strength and permeation properties, cement motar cubes of dimension 50 x 50 x 50 mm were cast (Fig. 3.5). Along with this, partial replacement of fine aggregate with slag (30% and 50%) was carried out. Hence, cement mortar cubes were cast containing slag as replacement along with the isolated bacteria in cement mixture. Mix proportions of slag and bacterial treatment in different sets of cube samples are shown in Table 3.2.

Table 3.2. Mixture proportions of cement motar cubes (per 50mm cube)

Composition		Control	Untreated		Bacterial Treated	
		C	30CS	50CS	30SBT	50SBT
Cement		76.2 g	76.2 g	76.2 g	76.2 g	76.2 g
Fine Aggregate		228.6 g	160.02 g	114.3 g	160.02 g	114.3 g
Slag	30% Replacement	-	68.58 g	-	68.58 g	-
	50% Replacement	-	-	114.3 g	-	114.3 g
Water		38.1 g	38.1 g	38.1 g	-	-
Bacterial Culture		-	-	-	38.1 g	38.1 g

*All experiments were conducted in triplicates.

Fig. 3.5. Preparation of cement motar cubes

3.3.2 Curing of Concrete Cubes

After casting, all the test specimens were stored at room temperature and were demolded after 24 hours. Further curing (Fig. 3.6.) was carried out as mentioned in table 3.3 and these specimens were used to study compressive strength and water absorption properties.

Table 3.3. Outline of different sets of concrete specimen and mechanism of curing treatment

Specimen		**Material**	**Mechanism of curing**
Control specimen		Cement:Sand Water/cement= 0.5	Water curing for 7 and 28 days
Steel slag motar specimen		Cement:Sand:Slag Water/cement=0.5	Water curing for 7 and 28 days
Steel slag and Bacterial mixed specimen	**30% replacement**	Cement:Sand:Slag Bacterial culture/cement=0.5	Submersion in NBU medium and Bacterial culture for 7 and 28 days
	50% replacement	Cement:Sand:Slag Bacterial culture/cement=0.5	Submersion in NBU medium and Bacterial culture for 7 and 28 days

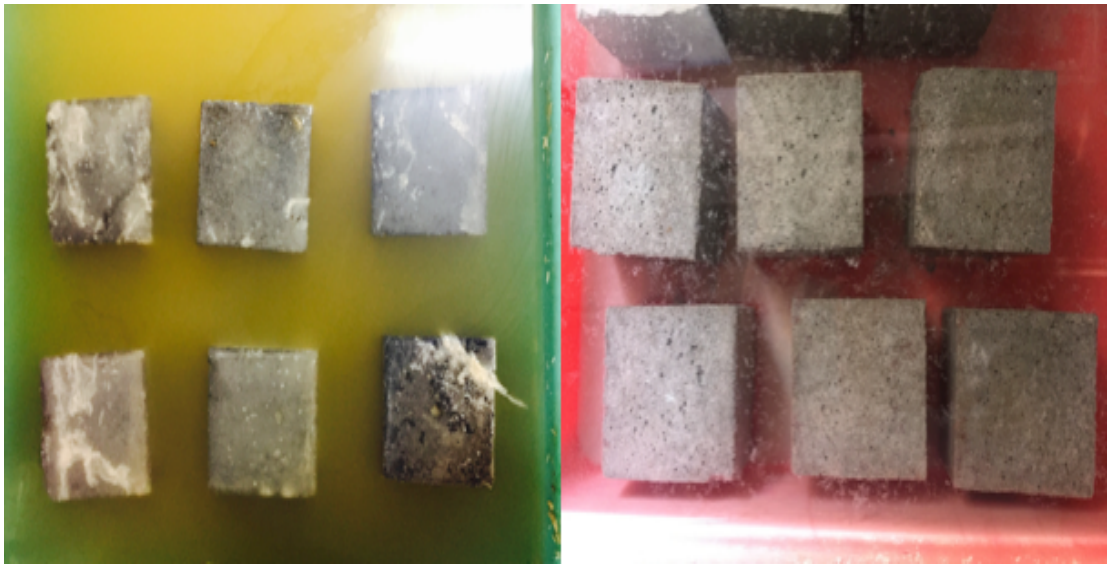


Fig. 3.6. Curing of cement motar cubes in NBU medium and water as specified in table 3.3

3.3.3 Testing of Compressive Strength

Compressive Strength of concrete cubes prepared with steel slag, without steel slag and bacteria treated cubes were determined at the age of 7 and 28 days as in BIS: 516 (1959). Specimens were placed on the bearing surface of Digital Compression Testing Machine (Fig. 3.7). The maximum load was then noted and the compressive strength was calculated as: Compressive strength (N/mm^2) = (Load in N/ Area in mm^2).

Fig. 3.7. Digital compression testing machine to test cube strength

3.3.4 Water absorption Test

For studying water absorption, bacterial treated and control blocks were dried in oven at 60^oC overnight and dry weight was measured ($W_{oven\ dried}$) as per IS 3495. To calculate the rate of moisture absorption, the blocks were immersed in water (Fig. 3.8) for different intervals of time (15 min, 30 min, 1h, 1.5h, 3h, 5h, 8h, 24h, 72h, 96h, 120h, 144h and 168h) and the weight of wet block was measured, after drying the surface with a towel. These were immediately put back in water after the weight was noted.

The sorptivity coefficient k [$cm.s^{-1/2}$] was calculated from the following expression

$Q/A = k\sqrt{t}$

Where, Q is the amount of water absorbed [cm^3]; A is the cross section of the cube with is in contact with water [cm^2]; t is the time [s]; Q/A is plotted against the square root of time, k is calculated from the slope of liner relation from these two.

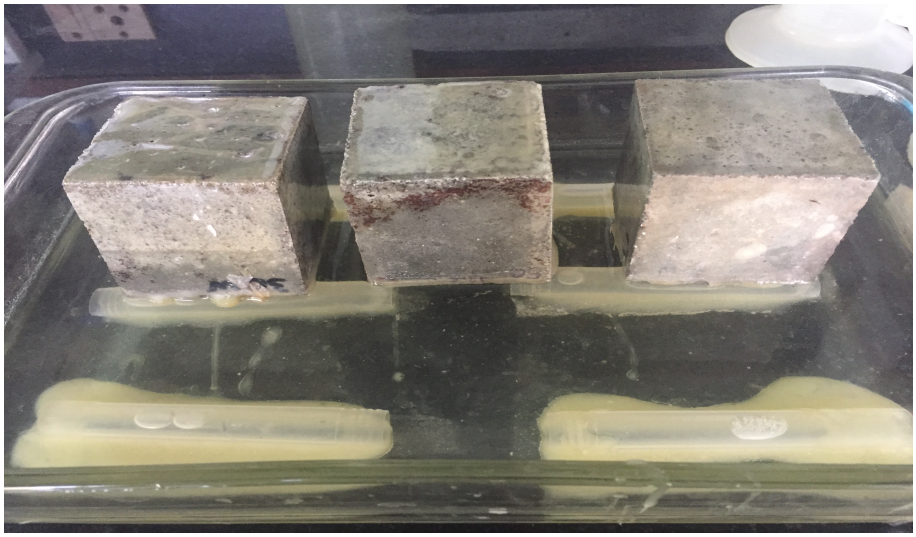


Fig. 3.8 Permeability analysis of motar cubes in a water tray

3.4. SEM-EDX Analysis

The morphology and chemical constituents of bacterial carbonate consolidated specimens were analyzed with Alizarin Red staining, SEM-EDX and XRD. Samples were completely dried at room temperature, and examined by SEM (Zeiss EVO50) at accelerating voltages ranging from 15 to 35 kV, which was equipped with an energy-dispersive X-ray analyzer (Bruker-AXS, QuanTax 200) for elemental analysis. Samples were gold coated with a sputter coating Emitech K575 prior to examination (Fig. 3.9). XRD-spectra were obtained using an X'Pert PRO diffractometer with a Cu anode (40 kV and 30 mA) and scanning from 3 to 60˚ 2θ. The components of the sample were identified by comparing them with standards established by the International Center for Diffraction data.

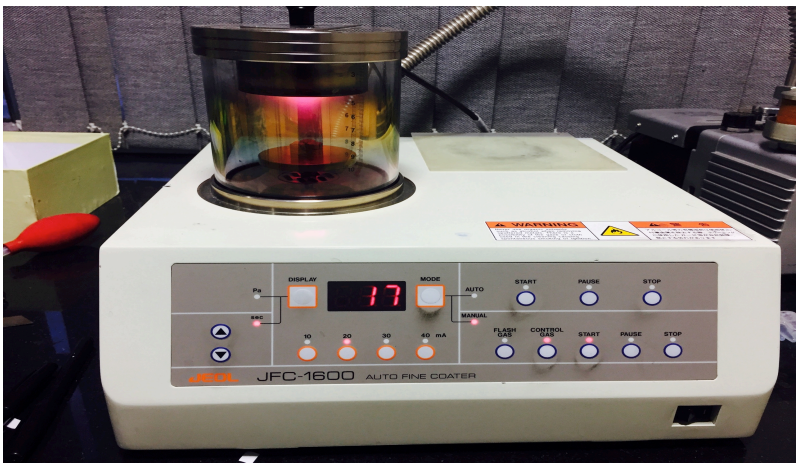


Fig. 3.9. Scanning Electron Microscope Coating apparatus

3.5 X- RAY DIFFRACTION (XRD) ANALYSIS

X-ray powder diffraction technique is the most prominent technique used for unraveling the structure of the materials in bulk and thin film forms. X-ray diffraction is a non-destructive technique used to determine the elements present in any particular substance. X-ray diffraction is based on the fact that, in a mixture, the measured intensity of a diffraction peak is directly proportional to the content of the substance producing it. The samples for X-Ray diffraction analysis were prepared in powdered form. Slag sample and slag bacterial treated specimen were taken and crushed into fine powder by pestle-mortar. The powder samples were analyzed in a powder X-ray diffractometer (PAN alytical X' Pro) (Fig. 3.10). The XRD spectrum were taken from 2Θ = 10°-80°. For a given sample, XRD graphs were obtained with intensities on Y-axis and 2Θ on X- axis. All the strong peaks in the XRD graph indicated the presence of the mineral.

Fig. 3.10. X-Ray diffraction instrument

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation and identification of calcifying bacteria

Although, numerous studies have investigated microbial diversity in wide range of habitats yet there are still many which await exploration. Ubiquitous nature of bacteria makes them ideal candidates for isolating them from even extreme environments and studying their diversity. Alkaliphilic ureolytic bacteria represent the most favoured pathway for the precipitation of carbonates. In the present study, ureolytic-calcifying bacteria was isolated from steel slag to exploit its MICCP property for further use in cement along with steel slag waste replacement.

4.1.1 Physical and chemical properties of steel slag

Steel slag is sub-angular to round in shape and greyish in color (Fig. 4.1). The pH value of steel slag was recorded as 12.2 representing its highly alkaline nature. The primary constituents of slag include aluminum, calcium, iron, magnesium, manganese, phosphate, silicon, and sulfur (Proctor et al., 2000). Shi et al. (2004) represented steel slag by a CaO–MgO–SiO_2–FeO quaternary system. However, the proportions of these oxides and the concentration of other minor components of slag are highly variable and can change from batch to batch even in one plant depending on raw materials, type of steel made as well as furnace conditions.

Fig. 4.1. Steel slag sample collected from Tata Steel Industry, Jamshedpur, India.

4.1.2 Isolation and enrichment of ureolytic bacterial species

Bacterial urease hydrolyzes the urea to form bicarbonate; ammonium and hydroxide ions, which further raises to pH and in turn results in the formation of carbonate ions in the presence of soluble calcium ions resulting in their precipitation as $CaCO_3$**.** Because of the carbonate precipitating ability of these alkaliphilic bacteria, alkaline property of slag of this site were investigated for isolation of calcifying bacteria. Dhami *et al* (2013) reported the biomineralization potential of alkaliphilic bacteria *Bacillus megaterium* SS3 that had been investigated on the basis of production of urease and precipitation of calcium carbonate.

Population of ureolytic bacteria differs according to the alkaline source. The isolated bacteria from steel slag sample were subjected to primary screening for the selection of efficient strain. The primary screening of the bacteria included a qualitative test of detecting the ability to utilize urea as an energy source. Bacterial isolates were screened for urease production in urease selective media, urea agar media (Fig. 4.2). Based on the pink color intensity out of twenty-four bacterial colonies, six were selected for further studies. These were designated as PP1, PP4, PP6, PP8, PP9, and PP11.

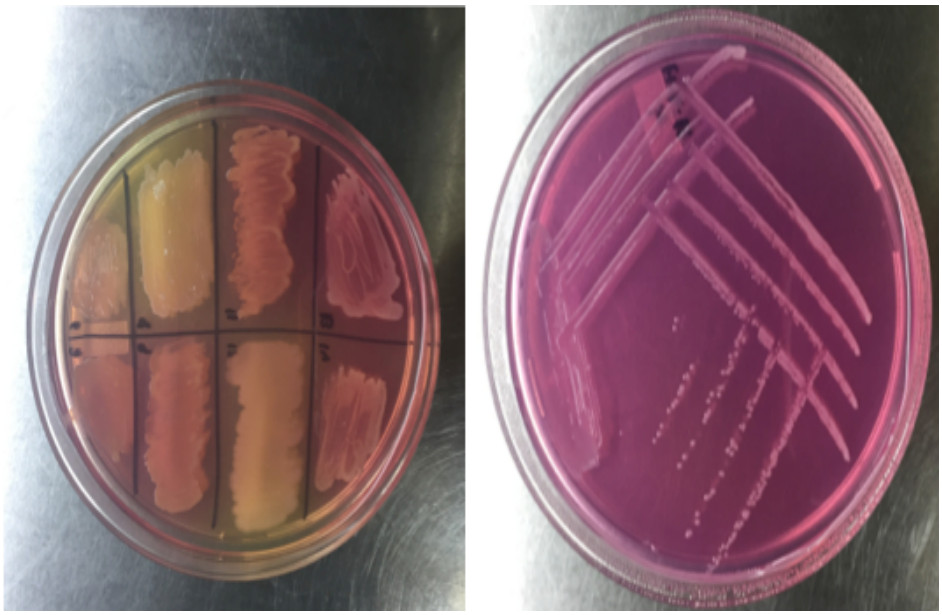


Fig. 4.2. Enrichment of ureolytic bacterial isolates on urea agar media.

4.1.3 Urease Assay

The selected bacterial isolates (PP1, PP4, PP6, PP8, PP9 and PP11) were selected to quantitatively note the urease activity at optimal conditions in presence of 2% urea at 37°C. Readings were recorded after regular intervals of 24 hrs. Bacteria are known to hydrolyze urea by urease mainly for the purposes of (1) increasing the ambient pH (Burne and Marquis, 2000), (2) utilizing it as a nitrogen source (Burne and Chen, 2001), and (3) using it as a source of energy (Mobley and Hausinger, 1989).

The highest productivities by isolate PP8, PP9, PP11 were observed at 96 hrs whereas it was 72 hrs for the isolate PP1, PP4, PP6 (Fig. 4.3) (Table 4.1). The urease productivity was observed to decrease with time. Among all the isolates, PP9 significantly produced higher urease activity. These isolates were selected for further study.

Similar results reported by Dhami et al. (2013) showed that urease production by *B. megaterium* SS3 increased significantly with time and maximum production was observed on the 4th day (650 U/ml) while after that, there was sharp decrease in the activity of the enzyme. Whiffin et al. (2004) suggested that in order to overcome the decrease in urease activity with time, the bacterial cells should be provided with fresh nutrient media so as to remove the repressive byproducts formed as a result of urea hydrolysis or the pH of the system should be maintained for optimizing the urease activity in case of *S. pasteurii*. A bacterium hydrolyzes urea by means of urease enzyme and increases the ambient pH, which either allows urea to be used as nitrogen source or as a source of energy (Mobley and Hausinger 1989; Burne and Chen 2001).

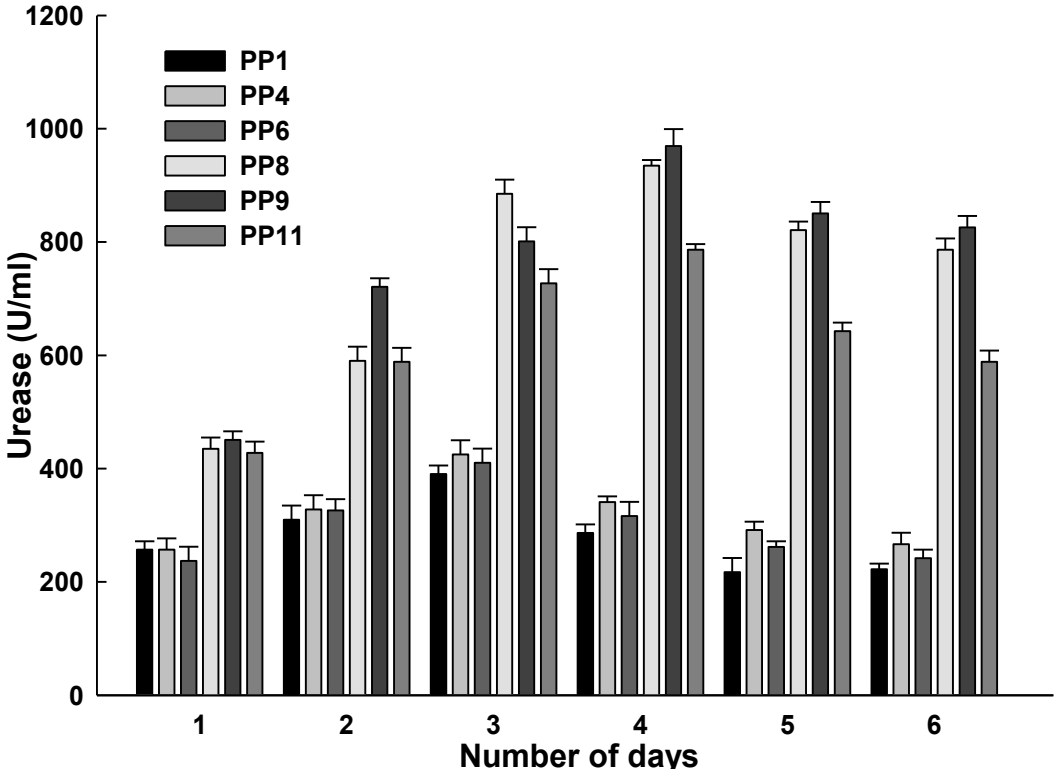


Fig. 4.3. Urease production (U/ml) by bacterial isolates from steel slag sample.

Table 4.1 Urease production by bacterial isolates.

Isolates	24 hr	48 hr	72 hr	96 hr	120 hr	144 hr
PP1	256.8±3.4^{d}	309.7±3.2^{d}	390.4±3.1^{f}	286.5±2.4^{f}	217.2±3.2^{f}	222.2±3.1^{f}
PP4	256.8±2.4^{d}	328.0±3.6^{c}	425.0±2.1^{d}	340.9±2.8^{d}	291.4±2.2^{d}	266.7±2.4^{d}
PP6	237.0±3.3^{e}	326.1±2.8^{c}	410.2±3.3^{e}	316.2±2.2^{e}	261.7±3.1^{e}	242.0±2.1^{e}
PP8	434.9±2.2^{b}	590.3±2.5^{b}	885.2±2.4^{a}	934.6±2.8^{b}	820.8±2.2^{b}	786.2±3.1^{b}
PP9	450.7±2.5^{a}	720.9±3.1^{a}	801.1±3.2^{b}	969.3±2.4^{a}	850.5±2.3^{a}	825.8±2.1^{a}
PP11	427.5±2.8^{c}	588.3±2.5^{b}	726.8±2.2^{c}	786.2±2.3^{c}	642.7±2.2^{c}	588.3±2.4^{c}

Mean values sharing the same letter within the column are not significant at $P<0.05$.
Values are mean ± SD (n = 3).

4.1.4 Molecular characterization of bacteria

After screening, six of the isolates were examined further. To identify these isolates, all six were subjected to 16S rRNA amplification using universal primers. About 1.5 Kb molecular sizes of amplicons were observed (Fig. 4.4). These were digested with restriction enzymes to obtain a distinct banding pattern (Fig. 4.5). It was observed that the isolates had more or less similar banding pattern. Since PP-9 exhibited the maximum urease activity, it was considered for characterization. 16S rRNA PCR product of PP9 isolate was cloned into pMDT20 vector. Plasmid DNA was extracted from this clone and amplified with M13-F and M13-R primers. MultAlin tool was utilized to align the sequences with closest organism. EZ Biocloud big data analysis platform was used to obtain the similarity of nearest related sequences and identify the organism.

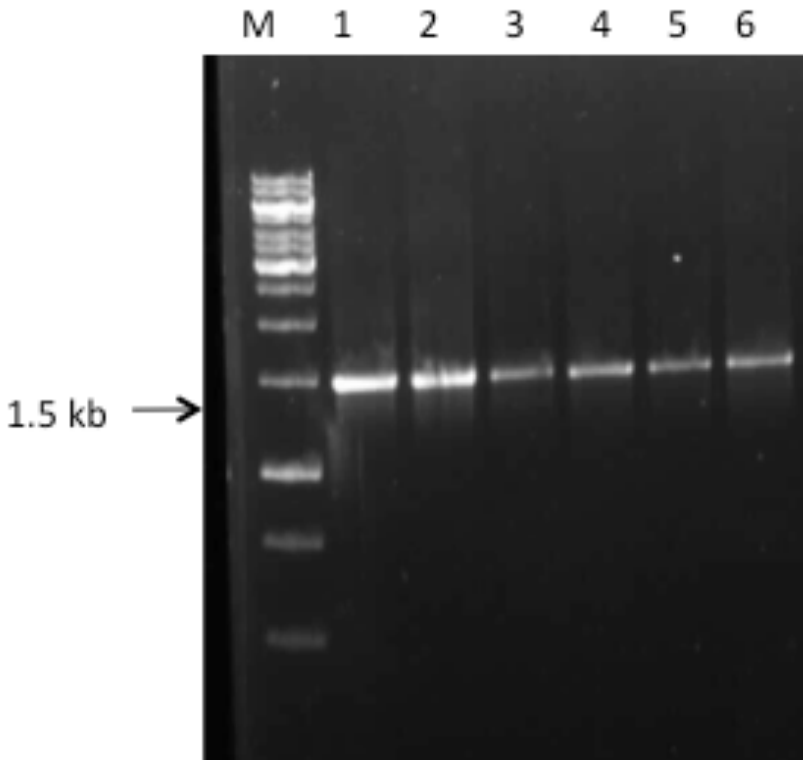


Fig. 4.4. 16S rRNA amplification of bacterial isolates lane 1-7: Marker, PP1, PP4, PP6, PP8, PP9, and PP11 respectively.

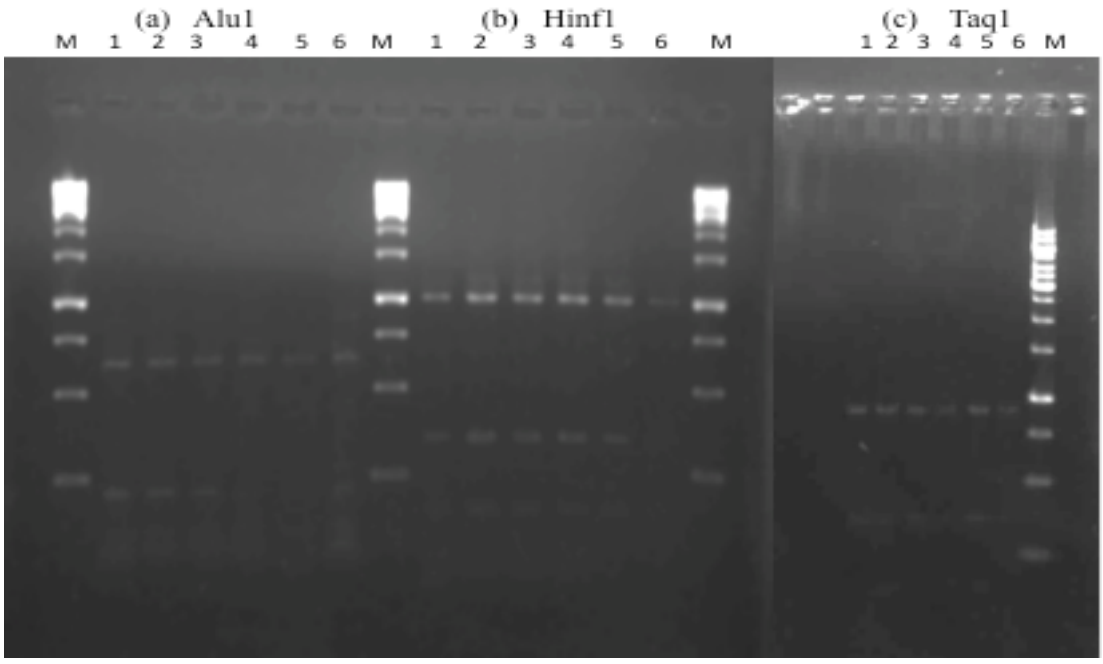


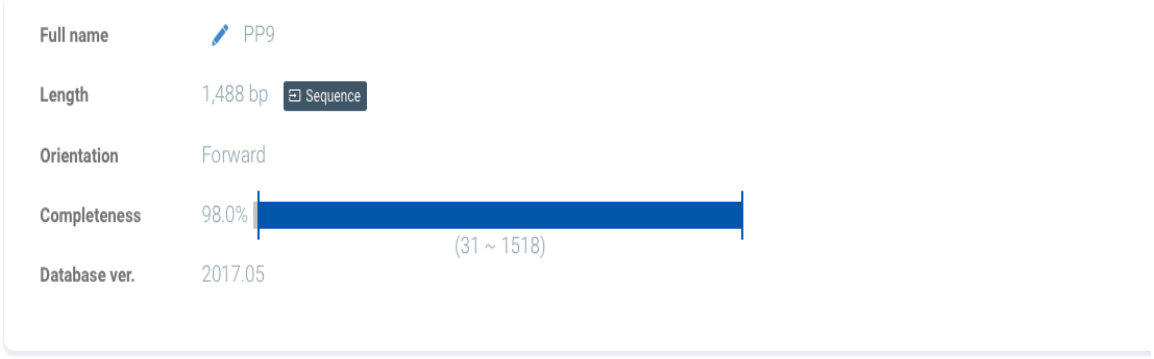
Fig. 4.5. Agarose gel electrophoresis of amplified 16S rDNA digested with restriction endonuclease (a) Alu I (b) Hinf1 and c) Taq I of bacterial isolates PP1, PP4, PP6, PP8, PP9 and PP11 respectively (Lane M: 1 Kb marker).

The sequence of the isolate PP9 as obtained on EZBiocloud was as follows;

>PP9
CAAGTCGAGCGAACAGATAAGGAGCTTGCTCCTTTGACGTTAGCGGCGGACGGGTGAGTAACACGTGGA
TAACCTACCTATAAGACTGAGATAACTTCGGGAAACCGGAGCTAATACCGGATAAGATTTTGAACCGCAT
GGTTCAATAGTGAAAGAAGGCCTTGATGTCACTTATAGATGGATCCGCGCCGTATTAGCTAGTTGGTAAG
GTAACGGCTTACCAAGGCAACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGAC
ACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAA
CGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATCAGGGAAGAACAAACGGGTAAGTA
ACTGTGCACGTCTTGACGGTACCTGATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATAC
GTAGGTGGTAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAATCTGATGTGA
AAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGAGGAAAGTGGA
AATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGT
CTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGT
AAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTTAGTGCTGCAGCTAACGCATTAAGCACTCCG
CCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCACACAAGCGGTGGAGCAT
GTGGTTTAATTCGAAGCAACGCGATGAACCTTACCAAATCTTGACATCCTTTGACCACTCTAGAGATAGA
GTTTTCCCCTTCGGGGGACAAAGTGACAGGTCGCGCATGGTTGTCGTCATCTCGTGTCGTGAGATGTTGG
GTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGTTGACT
GCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTACAC
ACGTGCTACAATGGACAATACAAAGGGCAGCTAAACCGCGAGGTCAAGCAAATCCCATATAGTTGTTCT
CAGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGTAGATCAGCATGCT
ACGGTGAATACTTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGCC
GGTGGAGTAACCATTTATGGAGCTAGCCGTCGAAGGTGGGACAAATGATTGGGGTGAAGTCGTAACAAG
GTAGCCGTATCGGATCCCCGGGTACCGAG

Phylogenetic analysis revealed that the bacterial isolate PP9 was related to Phylum Firmicutes (Fig. 4.6). The phylogenetic tree constructed with the sequences of representative bacteria revealed that PP9 clustered with four other members of *Staphylococcus pasteuri* species as shown in Fig 4.7. The complete sequence analysis of the isolate PP-9 was done and it was identified as *Staphylococcus pasteuri* as it showed 98.94% resemblance with it.

Mclean *et al* (1986) reported that the urease is located in the membrane and periplasm of *S. warneri* and *S. hominis*, which are more closely related to *S. pasteuri*. All of the three also exhibit the capacity of growing at 15-45^{o}C. They also produce catalase and urease and lead to acid production (aerobically) utilizing several substrates (glycerol, D-glucose, sucrose, and -D-fructose).



List of hits from EzBioCloud 16S database

Select hits by database

All | Valid names only | Excel | FASTA | EzEdito

Tasks	Hit taxon name	Hit strain name	Accession	Similarity	Diff/Total nt	Hit taxonomy	Completen
⇄ O	Staphylococcus pasteuri	ATCC 51129(T)	AF041361	98.94	15/1418	Bacteria;Firmicutes;Bacilli;Bacillales;Staphylococcaceae;Staphylococcus	96.2
⇄ O	Staphylococcus warneri	ATCC 27836(T)	L37603	98.61	20/1438	Bacteria;Firmicutes;Bacilli;Bacillales;Staphylococcaceae;Staphylococcus	99.7
⇄ O	Staphylococcus petrasii subsp. petrasii	CCM8418(T)	JX139845	98.13	27/1442	Bacteria;Firmicutes;Bacilli;Bacillales;Staphylococcaceae;Staphylococcus;Staphylococcus petrasii	100.0
⇄ O	Staphylococcus petrasii subsp. jettensis	SEQ110(T)	JN092118	98.00	28/1402	Bacteria;Firmicutes;Bacilli;Bacillales;Staphylococcaceae;Staphyloc	97.5

Fig. 4.6. PP9 sequence showing similarity to strains in EZ Biocloud.

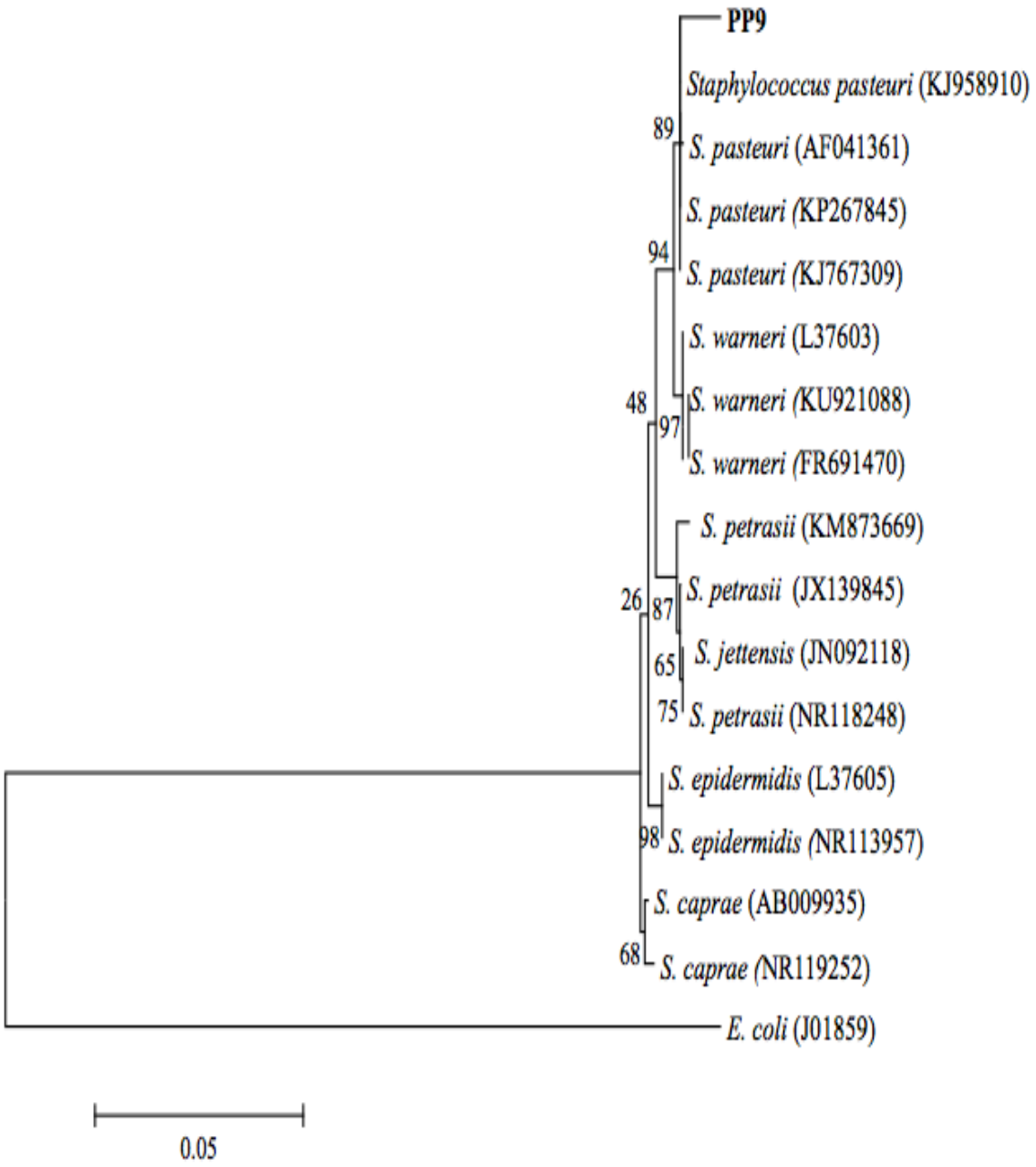


Fig. 4.7. Neighbor-joining tree based on bacterial 16S rRNA sequence data of PP9 of current study along with sequences available in GenBank database. Numerical values indicate bootstrap percentile from 1000 replicates. *Escherichia coli* was used outgroup taxa.

4.1.5 Morphological characterization of bacterial isolate PP9

The bacterial strain PP9 was found to be gram-positive cocci, which formed an opaque creamy colony without any pigmentation on nutrient agar. The bacterial isolate was able to grow between pH 7 and 11. The description of PP9 was in accordance to previous report where S. *pasteuri* cells were identified as non-sporulating, non-motile, gram-positive cocci, 0.5 to 1.5 micrometer in diameter, occurring singly, in pairs, in tetrads, or in clusters (Chesneau et al., 1993).

The growth profile of PP9 was studied upto 30 hrs in nutrient broth as shown in Fig. 4.8. The cell growth was noted upto 30 hrs at intervals of 2 hrs. Optical density was measured to determine the cell concentration in the media. The growth curve showed that the cells started to grow after a time interval of 4 hours and continued to grow upto 24 hrs. Hence a longer exponential phase and shorter lag phase was recorded**.**

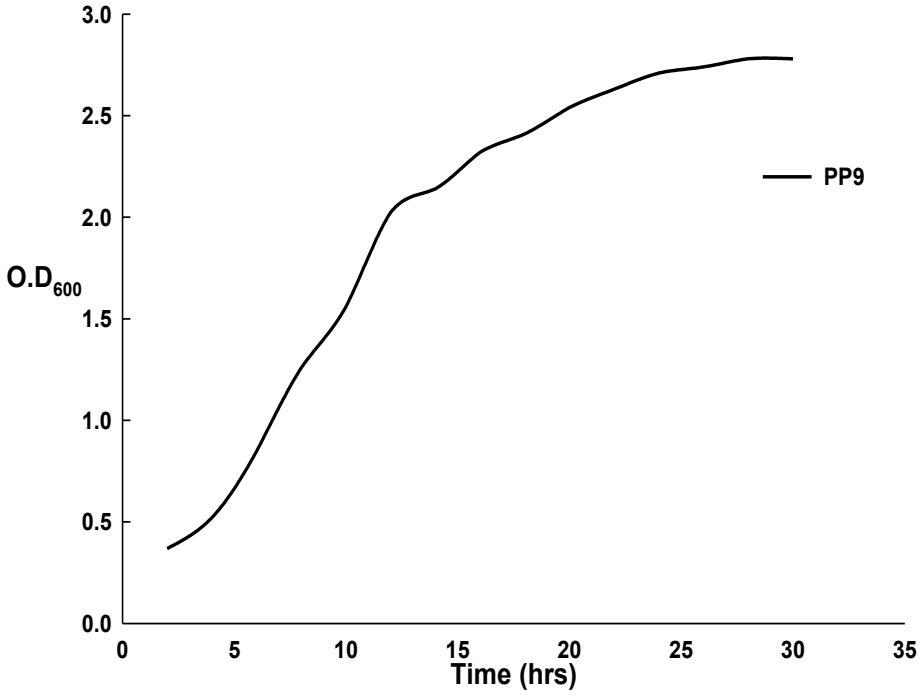


Fig. 4.8 Growth curve of PP9 in Nutrient Broth Medium

4.2 Pathogenicity of the isolated bacteria

To study the pathogenicity of PP9, coagulase test and mannitol test were carried out. *Staphylococcus pasteuri* is a coagulase-negative, Gram-positive organism (Savini et al., 2009). *Staphylococcus sciuri, S. warneri, S. pasteuri* and *S. xylosus* are nonpathogenic species and its presence is usually considered as safe (Marino et al., 2011). Species of coagulase-negative staphylococci (CNS) are considered technologically important in the manufacturing processes of various meat-derived products.

On the other hand, *Staphylococcus aureus* is well known for their implications in human health diseases (Yugueros et al., 2000). *Staphylococcus aureus* is a foodborne pathogen that is considered one of the world's leading causes of disease outbreak related to food consumption, which often cause food-related illness (Greig et al., 2007). Kateete et al. (2010) evaluated common identification methods for *S. aureus* aiming at improving the diagnosis through a combination of available phenotypic methods. It was reported that the efficiency of the tube coagulase test could be markedly improved by sequel testing of the isolates with Mannitol salt agar.

When the coagulase test was carried out, clot formation was not observed (Fig. 4.9), which indicated the absence of the organism *Staphylococcus aureus.* Hence PP9 was identified as a non-pathogenic species by coagulase tube test.

Sequel testing of pathogenicity for PP9 was carried out on mannitol agar media. *S. aureus* causes fermentation of mannitol, which results in the formation of yellow zones around the colonies. In current study, absence of these zones was seen. The observed pink colonies surrounded by red zones indicated a negative result as shown in Fig 4.10. This further supported the coagulase negative result and PP9 isolate was confirmed to be non-pathogenic.

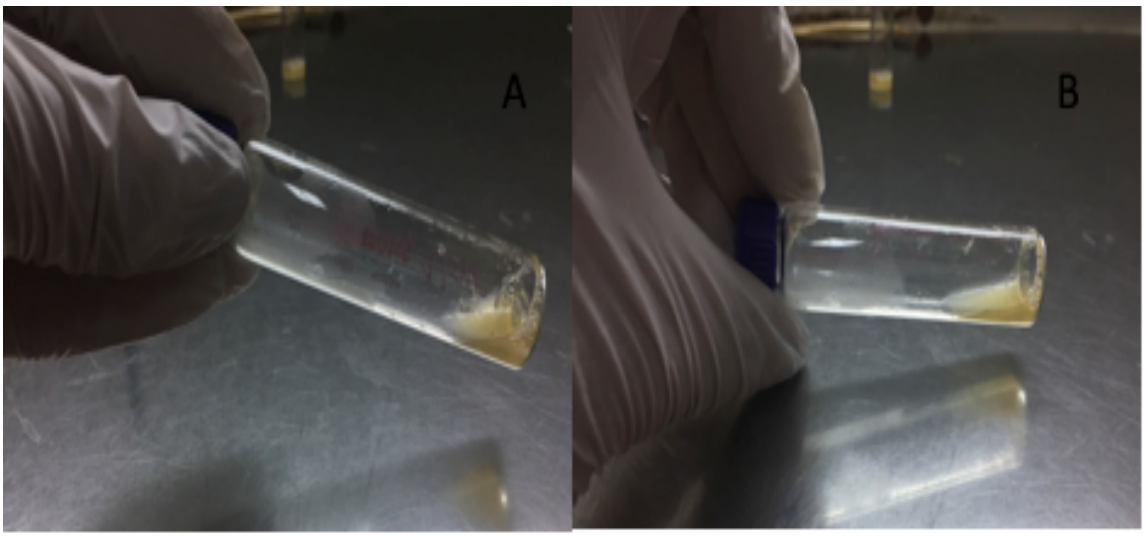


Fig. 4.9. Coagulase test of bacterial isolate PP9 using Hiaureus™ Coagulase Confirmation kit A) Tube inoculated with bacterial isolate; B) After incubation no clot formation was observed indicating a negative result.

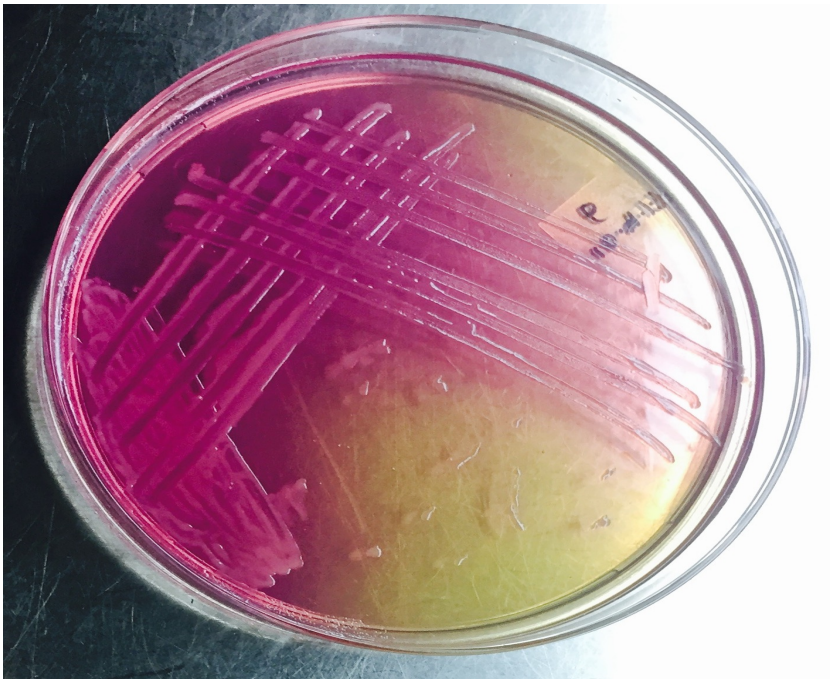


Fig. 4.10. Growth of bacterial isolate PP9 on Mannitol Agar Media.

4.3 Estimation of pH variations and soluble calcium content at flask level

Ureolytic bacteria produce ammonia as a result of enzymatic urea hydrolysis and create alkaline microenvironment around the cell in the nutrient broth medium. 100ml of NB medium with 2% urea was inoculated with 1% overnight grown culture. The variations in pH and soluble calcium were observed over a period of 144 hrs (Table 4.2). Consumption of available calcium content in medium resulted in initial decrease in pH. After 5 days, increase of pH was observed due to the presence of ammonium ions and additional release of CO_2 from enzymatic urea hydrolysis, which accelerates the rate of urease induced carbonate precipitation (Fig. 4.11). So, an active participation of bacterial urease is necessary for carbonate precipitation. The dissolved CO_2, in the form of carbonate or bicarbonate , not only precipitates as part of calcite but also functions as buffer, which slows down the pH increase as ammonia is produced from urea hydrolysis (Stock-Fischer et al., 1999).

Soluble calcium content estimation by the bacterial isolate PP9 was evaluated at flask level by EDTA titration method (Table 4.3). Initial readings of soluble calcium were noted to be the highest. The soluble calcium content was found to decrease with time due to its consumption by calcifying bacteria (Fig. 4.12). This also is an indicator of calcium carbonate precipitation as soluble calcium content is converted to calcium carbonate crystals by our isolate PP9. Similar observation by Dhami et al. (2013) reported the Ca^{2+} concentration in the supernatant of ureolytic bacterial culture reduced with time while the pH of the culture filtrate increased leading to precipitation of $CaCO_3$.

Table 4.2. Variation in pH of medium inoculated with PP9.

Time (hrs)	pH
24	11.9 ± 0.3^{a}
48	11.3 ± 0.6^{ac}
72	10.8 ± 0.4^{bc}
96	10.1 ± 0.2^{b}
120	9.8 ± 0.5^{b}
144	10.6 ± 0.5^{ab}

Mean values sharing the same letter within the column are not significant at $P<0.05$. Values are mean ± SD (n = 3).

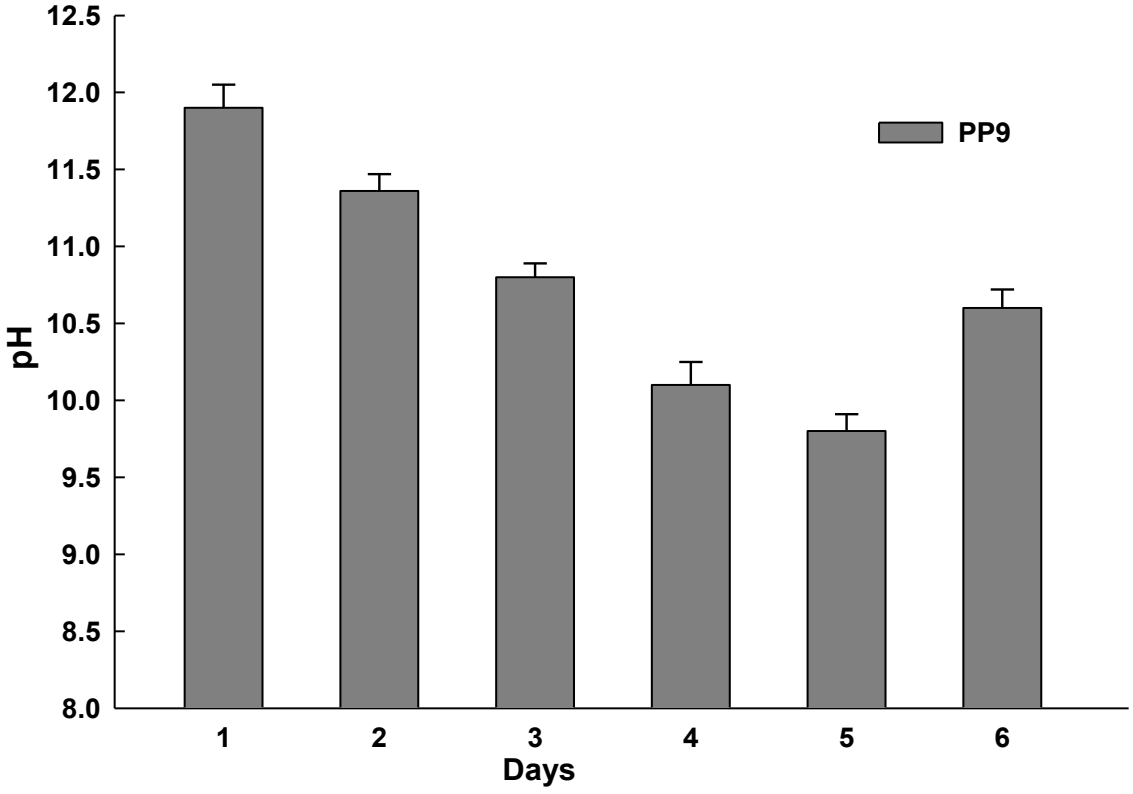


Fig. 4.11. Variation in pH of medium inoculated with PP9

Table 4.3. Variation in soluble calcium content (mM) of medium inoculated with PP9.

Time (hrs)	Soluble Calcium (mM)
0	93.7 ± 1.6^{a}
24	87.5 ± 1.9^{b}
48	75.0 ± 2.1^{c}
72	68.7 ± 1.7^{d}
96	56.7 ± 1.6^{e}
120	50.0 ± 1.2^{f}
144	50.0 ± 1.4^{f}

Mean values sharing the same letter within the column are not significant at $P<0.05$. Values are mean ± SD (n = 3).

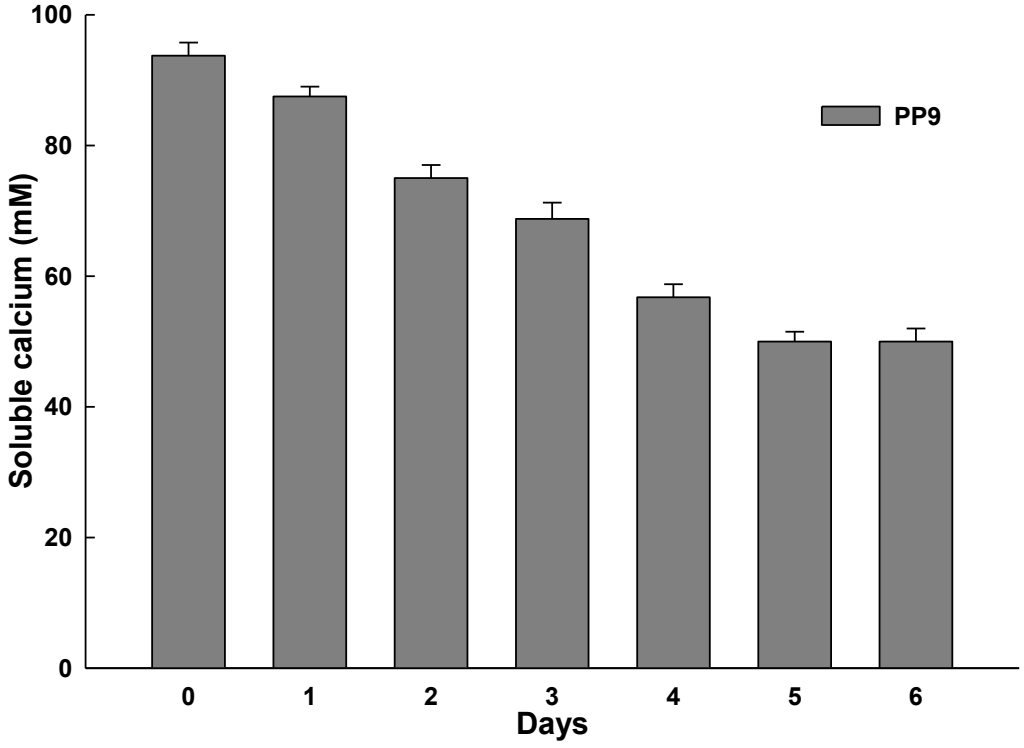


Fig. 4.12. Variation in soluble calcium content of medium inoculated with PP9

4.4 Durability and permeation studies of cement motar cubes

In our present study apart from fine aggregate replacement with steel slag, effect of bacterial treatment was studied. Mechanical and permeation properties of cement motar specimens were analyzed. No substantial work has been reported till date in accordance to our current study, which combined MICCP technology along with slag replacement.

To study the compressive strength enhancement due to slag replacement and bacterial treatment, specimens were cured for 7 and 28 days. Water absorption properties were tested after curing for a period of 28 days. Biocementation was visualized clearly on the surface of PP9 treated motar cubes after 28 days of curing as shown in (Fig. 4.13). These specimens were used after curing for further analysis of mechanical strength and permeation properties.

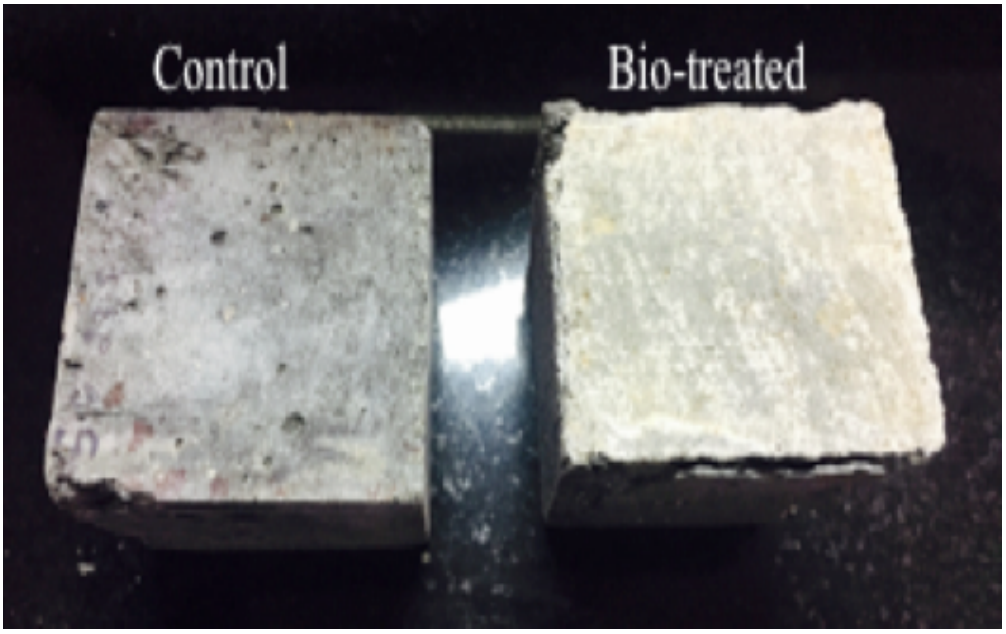


Fig. 4.13. Cement Motar cube control and bio-treated sample

4.4.1 Testing of Compressive Strength

To study the effect of bacterial treatment and slag replacement, compressive strength of samples was analyzed after 7 and 28 days curing as shown in table 4.4. Control showed minimum compressive strength value out of all the specimens. When compared to control, no significant change in strength was observed in 30CS and 50CS specimens after 7 day curing. However the bacterial treated specimens along with slag replacement showed notable change in strength after 7 day curing. 30CS showed the highest strength gain followed by 50CS as compared to the control specimen. At the age of 28 days, it was observed that there was a 28% increase in compressive strength of 30SBT specimen as compared to control. However, 50SBT specimen showed only 15% increase in strength when compared to control (Fig. 4.14).

In a previous study, Achal et al. (2011) reported measurable increase in compressive strength by application of *S. pasteurii* in cement mortar cubes. At 28 days, it reported that there was a 36% improvement in the compressive strength of mortar specimens with bacterial cells compared with control.

In present study, reduction in compressive strength is observed as the content of steel slag was increased. Although 30 SBT showed the highest gain in compressive strength yet as the amount of steel slag replacement was increased from 30% to 50%, overall strength decreased. This result was in accordance with a previous study conducted by Li et al. (2009) which reported that compressive strengths of concrete with steel slag replacement tends to decrease as the content of steel slag increases. Characteristics of steel slag aggregate concrete may be attributed to the impervious nature of the steel slag aggregate (Proctor et al., 2000).

Table 4.4. Compressive strength (MPa) of different specimens.

Specimen	7 Days	28 Days
Control	20.6 ± 1.2^{bc}	24 ± 1.1^{b}
30CS	21.1 ± 1.4^{abc}	27.3 ± 1.3^{ab}
50CS	20.26 ± 1.6^{c}	26.3 ± 1.4^{b}
30SBT	24.5 ± 1.6^{a}	30.8 ± 1.9^{a}
50SBT	24.4 ± 1.7^{ab}	27.8 ± 1.7^{ab}

Mean values sharing the same letter within the column are not significant at $P<0.05$. Values are mean ± SD (n = 3).

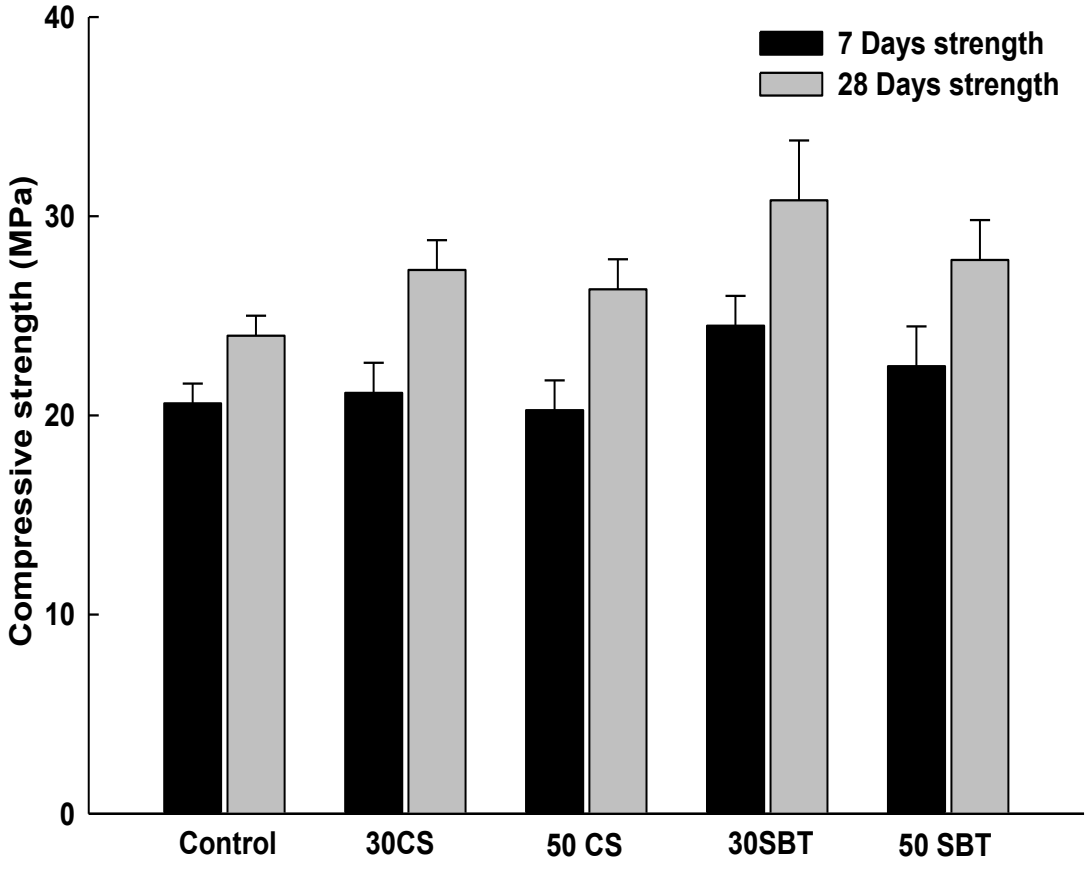


Fig. 4.14. Compressive strength analysis of motar cube specimens

4.4.2 Water absorption Test

Decrease in permeability of cement motar cubes treated with bacteria along with 30% (30SBT) and 50% (50SBT) replacement was observed. Fig. 4.15 shows the effect of calcite precipitation and replacement on the water absorption. The cubes were cured in bacterial inoculated NBU medium over a period of 28 days and the water absorption property was studied for over 96 hours. Among all the specimens, control specimen showed highest water absorption.

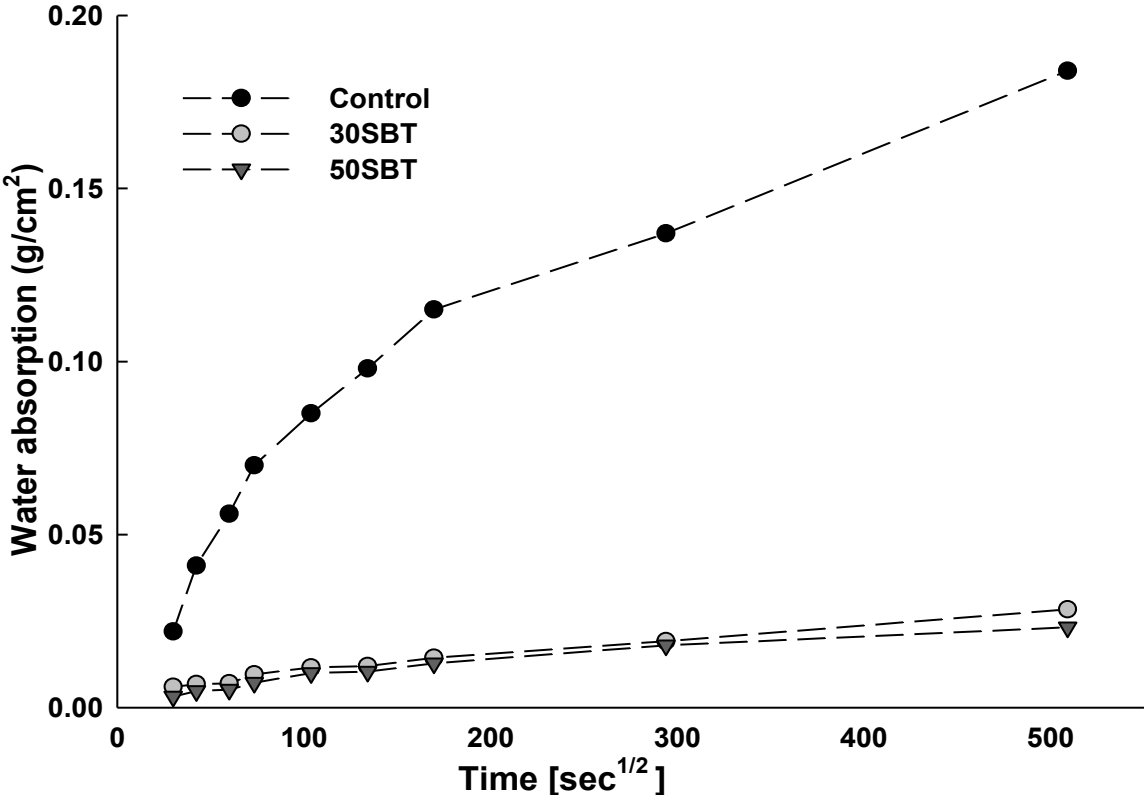


Fig. 4.15. Capillary water absorption of different motar cubes

It was concluded that the bacterially treated specimen 50SBT absorbed nearly eight times less water as compared to the control specimen. Almost similar trend of water absorption was observed in 30SBT specimen. Our results were in accordance with study conducted by Achal et al. (2011) which reported the motar cubes treated with bacterial cells (Bp M-3) absorbed nearly five times less water than the control cubes over a period of 168 h.

The reduction in the extent of water absorption may be attributed to the fact that due to bacterial calcification, calcium carbonate crystals were deposited. This reduced the pores on the surface of the cement motar cubes leading to protective film coating, which prevented ingress of water. As reported by De Muynck et al. (2010), large amounts of pores are plugged due to precipitation, the pore connectivity and water accessible porosity is decreased immensely. Achal et al. (2010) observed significant decrease in water absorption rate in cement mortar cubes upon treatment with bacteria as compared to control.

In present study, deposition of a layer of calcium carbonate crystals on the surface of motar cubes by PP9 in presence of slag replacement was clearly visible. The evidence of microbial calcite precipitation examined under a scanning electron microscope (SEM) is shown in microstructural analysis section. MICCP has the potential to improve the resistance of cementitious materials towards water ingress processes. As a consequence, the ingress of aggressive agents may also be limited.

Since a similar water absorption trend was observed in 50SBT and 30SBT specimens, it could be concluded that major reduction in water absorption occurred due to the bacterial calcium carbonate precipitation. Whether the replacement was 30% (30SBT) or 50% (50SBT), it did not play a significant role in improving the permeation property. However, high increase in compressive strength was noted in 30SBT specimen as compared to the insignificant change of strength in 50SBT specimen. It was concluded that the 30SBT specimen were best suited to exhibit the effective improvement in mechanical strength and permeation properties studied in motar cubes treated with our selected PP9 isolate.

Overall, application of MICCP technology along with the slag replacement in presence of PP9 proved to an efficient technique to work on improving the strength and permeation properties in concrete technology. Since the bacteria was isolated from a highly alkaline environment, it showed high efficacy in high pH conditions prevalent in cement as well.

4.5 Microstructural Analysis

In order to further determine the presence of carbonate crystals, specimens from the flask level experiment and cement motar cubes were subjected to SEM, EDX and XRD analysis. Cement motar cubes (30CS and 50CS) with slag replacement were subjected to SEM (Fig. 4.16), which showed presence of irregular shape particles and no precipitation. SEM and EDX analysis of untreated steel slag particles (Fig 4.17 A and B) and PP9 bio treated slag (Fig 4.17 C and D) at flask level was taken. Untreated slag particles were observed with high content of calcium and iron and relatively low quantity of magnesium, copper, silicon and aluminum were seen as well (Fig. 4.17 B). SEM images of bio treated slag clearly depicted the formation of calcium carbonate crystals in the presence of PP9 as shown in Fig. 4.17 C. At few regions PP9 cells were observed to be embedded within the carbonate crystals while at other regions they were seen in close proximity of the crystals. The presence of calcium carbonate crystals associated with bacteria depicts that PP9 served as nucleation sites during the mineralization process. These crystals were mainly vaterite and rhombohedral in shape. Its EDX analysis revealed peak of high calcium and carbon as shown in Fig. 4.17 D.

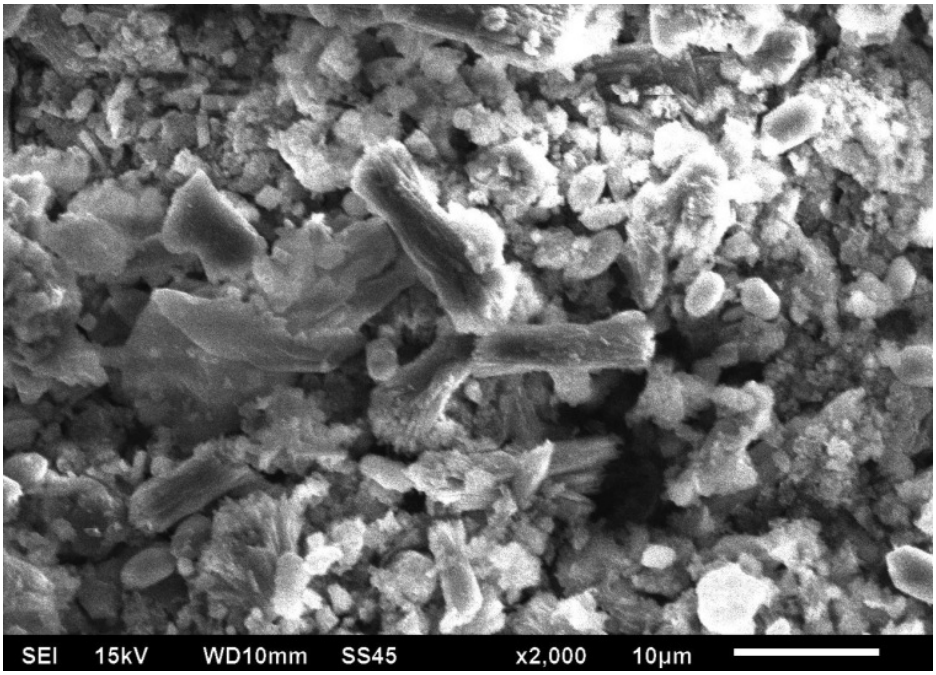


Fig. 4.16. Scanning electron micrograph of cement motar cube

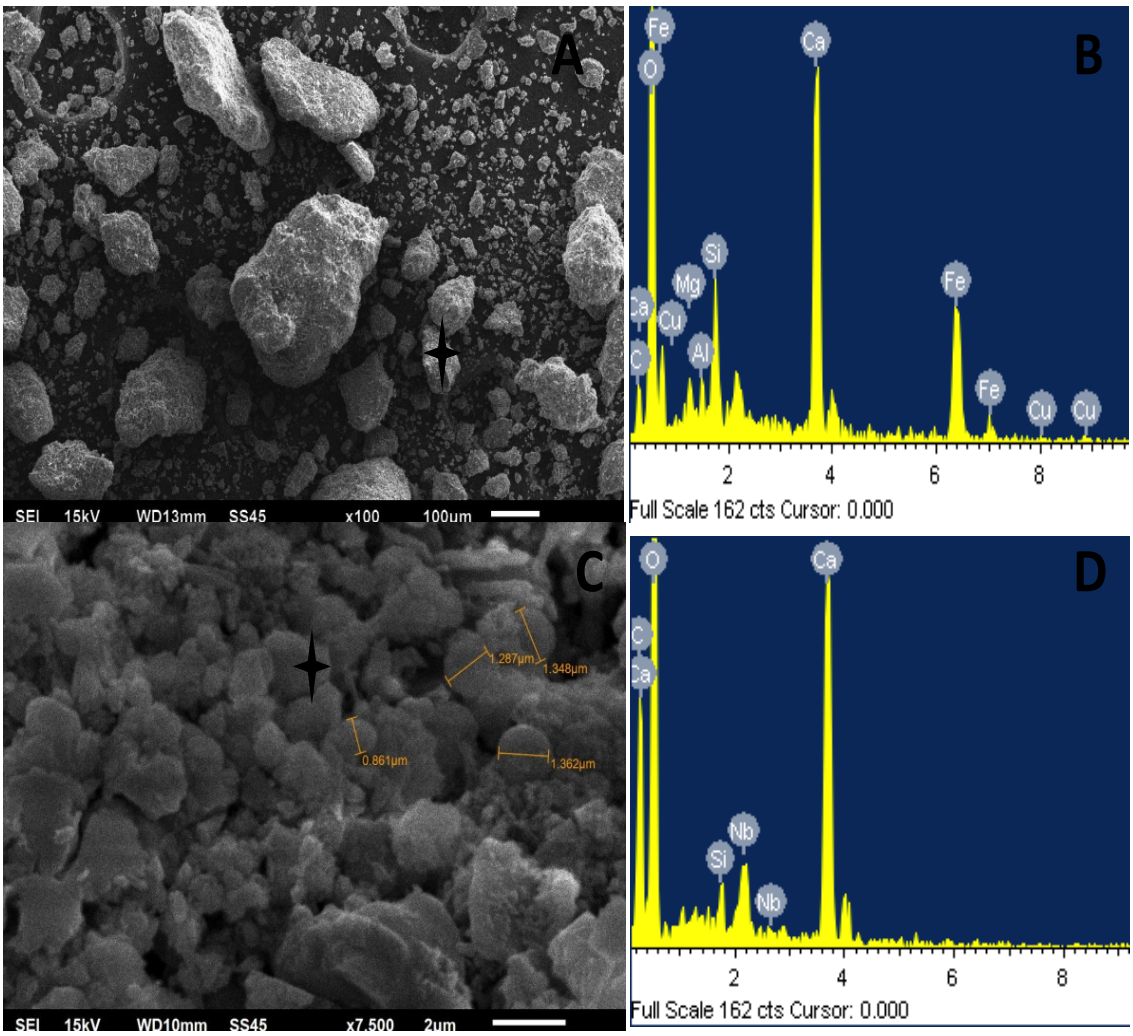


Fig. 4.17. Scanning electron micrograph and Energy dispersive X ray spectrum of slag sample (A, B) and PP9 treated slag sample (C, D)

In a previous study Dhami et al. (2013) reported that once super-saturation is achieved, calcium carbonate formation by heterogeneous nucleation readily occurs on the bacterial surfaces. Rhombohedral and spherical crystals associated with bacterial cells were characterized as calcite and vaterite. Stock Fischer et al. (1999) reported that once the initial calcite precipitates on the nucleation sites, the crystals continue on growing due to presence of microbial activities.

Scanning electron micrograph and Energy dispersive X ray spectrum of 50SBT (Fig. 4.18 A and B) and 30SBT (Fig. 4.18 C and D) cement motar cubes, with Steel slag and bacterial treated specimen was studied. Distinct calcite and vaterite crystals were observed in close proximity of the bacterial cells. EDX analysis revealed high peaks of calcium and carbon in 50SBT as shown in Fig. 4.18 B. Similar EDX peaks were observed in case of 30SBT with slightly less amount of calcium as shown in Fig. 4.18 D.

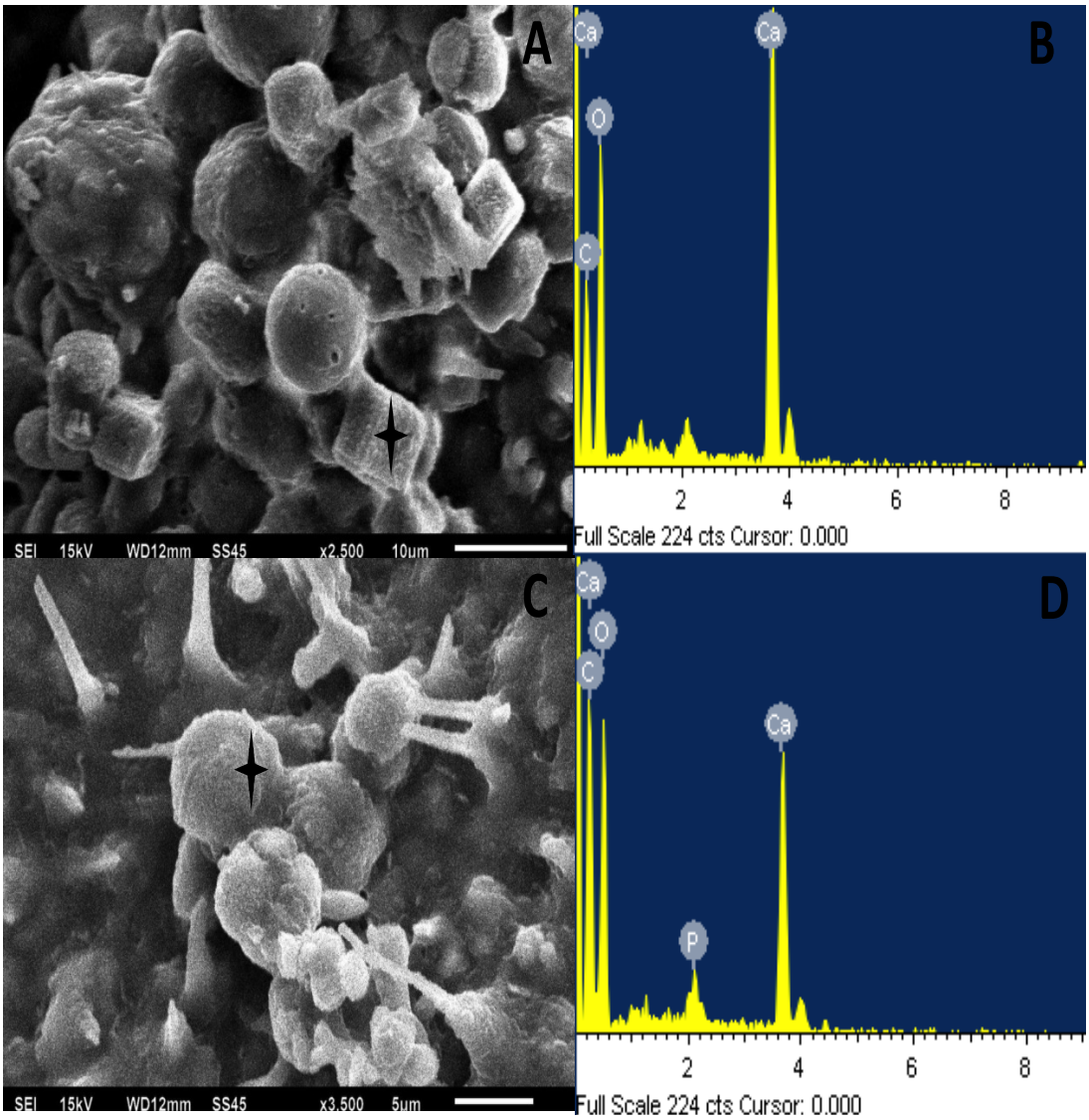


Fig. 4.18. Scanning electron micrograph and Energy dispersive X ray spectrum of 50SBT (A, B) and 30SBT (C, D)

Three crystal forms of $CaCO_3$ nucleation with same chemical formula and different structure are calcite, aragonite and vaterite. Calcite is considered as the most stable form of $CaCO_3$, with simple rhombohedral shape (De Yoreo & Vekilov, 2003). Ruiz-Agudo et al. (2012) reported that carbonate polymorph formation is dependent on the type of substrate present for bacterial attachment. They demonstrated that calcite substrates favor bacterial attachment and promote the formation of abundant calcite while under the same culture conditions; vaterite spheres are formed on silicate substrates.

The mineral constituents of both untreated slag sample (Fig. 4.19 A) as well as bacterial treated slag (Fig. 4.19 B) were then investigated by XRD analysis. High amount of iron and silicate was observed in untreated slag sample while high amount of calcium carbonate in the bacterial treated slag sample along with other components of slag. This further proved the formation of calcium carbonate in case of bacterial treated slag specimens.

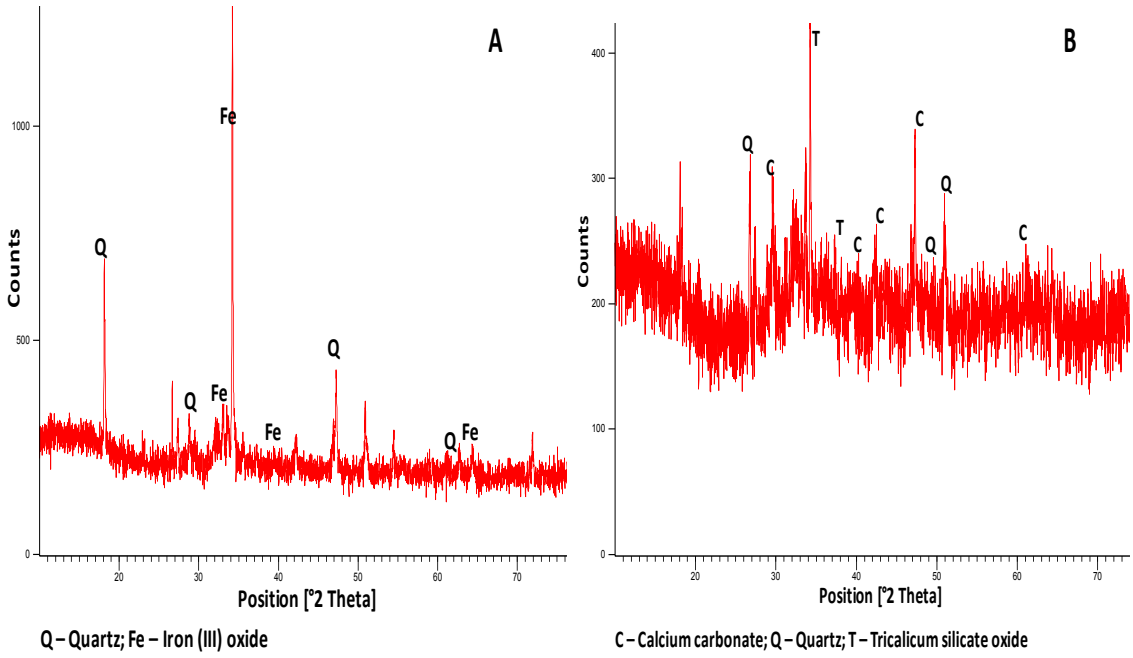


Fig. 4.19. X ray diffraction analysis of A) Slag sample and B) PP9 treated slag sample

Conclusion

In the current study, microbial cementitious structure was prepared with incorporation of steel slag at lab scale. In order to outlive the highly alkaline nature of steel slag and cement, a ureolytic bacteria with high urease activity was isolated from steel slag. Isolated bacteria designated as PP9 showed highest urease activity and exhibited maximum calcium carbonate precipitation. Phylogenetic analysis of the isolate PP9 showed homology of 98.94% with *Staphylococcus pasteruii*. Non-pathogenicity of PP9 was confirmed on the basis of mannitol salt agar and coagulase test. Steel slag was used as a replacement of fine aggregate in cement motar cubes in the ratio of 30% and 50%. Among all the specimens, 30SBT specimen was concluded to exhibit the highest strength gain of 28% and the water ingress was reduced by eight times as compared to the control specimen. SEM analysis endorsed the involvement of bacterial cells in calcium carbonate precipitation. Polymorphs of calcium carbonate crystals were identified as calcite and vaterite.

Pilot study of waste replacement along with microbial treatment was successfully evaluated at lab scale. In conclusion, this study paved the way for sustainable production of eco-friendly building material with enhanced durability. Outcome of the current study can be implemented from lab scale to field scale.

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Appendix I

A) Nutrient broth

Ingredient	Quantity (g/l)
Peptone	5
Beef extract	1.5
Yeast extract	1.5
Sodium chloride	5

Boiled to dissolve the medium completely, sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min, pH 7.4 ± 0.2

(B) Nutrient Broth – Urea Media (NBU)

Ingredient	Quantity (g/l)
Peptone	5
Beef extract	1.5
Yeast extract	1.5
Sodium chloride	5
Urea	20

The pH of the media was adjusted to 6.5 with 1 N HCl prior to autoclaving without urea. Filter- sterilized urea was added later. The final pH was adjusted to 8.0 by adding 1N HCl.

(C) Nutrient Broth – Urea $CaCl_2$ Media (NBUC)

Ingredient	Quantity (g/l)
Peptone	5
Beef extract	1.5
Yeast extract	1.5
Sodium chloride	5
Urea	20
$CaCl_2$	25mM

The pH of the media was adjusted to 6.5 with 1 N HCl prior to autoclaving without urea and $CaCl_2$. Filter-sterilized urea and $CaCl_2$ was added later. The final pH was adjusted to 8.0 by adding 1N HCl.

(D) NA agar plates

Prepared NB broth as above, added agar (15 g/l), autoclaved, and cooled. Poured plates and stored at 4°C

(E) Luria-Bertani (LB) Medium

Ingredient	Quantity (g/l)
NaCl	10.0
Beef extract	5.0
Tryptone	5.0
Agar	10.0

pH adjusted to 8.0 with 1N NaOH, sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min, added filtered ampicillin 50 µg/ml to prepare LB - Ampicillin plates

(F) LB/amp+ agar plates
Prepared LB broth as above, added agar (15 g/l), autoclaved, and cooled to 50°C, added ampicillin 50 µg/ml, Poured plates and stored at 4°C

(G) Mannitol Salt Agar

Ingredient	Quantity (g/l)
D-Mannitol	10
Beef extract	1
Peptone	10
NaCl	75
Agar	15
Phenol red	0.025

(H) IPTG stock solution (0.1M)
1.2 g IPTG Added water to 50 ml final volume, filtered and stored at 4°C

(I) X-Gal (2ml)
100 mg 5-bromo, 4 chloro, 3-indolyl, D galactoside dissolved in 2ml N,N-dimethylformamide, covered with aluminum foil and stored at 20°C

(J) LB plates with Ampicillin/IPTG/X-Gal
Prepared LB plates with ampicillin as above; 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X- Gal spread over the surface of LB ampicillin plate and allowed to absorb for 30 minutes at 37°C prior to use

(K) Agarose Gel Loading Dye (6X)

Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
Glycerol in water	30.0%

(L) Primers

M13 forward primer	5''-GTAAAACGACGGCCAGT-3'
M13 reverse primer	5'-CAGGAAACAGCTATGAC-3
16S rDNA forward primer	5'-ACGGGCGGTGTGTTC-3'
16S rDNA reverse primer	5'-IIIICGICGICATCIGGC -3'

Appendix II

Buffers used in Plasmid Isolation:

Buffer	Composition
Buffer P1 (resuspension buffer)	50 mM Tris·Cl, pH 8.0 10 mM EDTA 100 µg/ml RNase A
Buffer P2 (lysis buffer)	200 mM NaOH, 1% SDS (w/v)
Buffer N3 (neutralization buffer)	3.0 M potassium acetate pH 5.5
Buffer PB (wash buffer)	1.0 M NaCl 50 mM MOPS, pH 7.0; 15% isopropanol (v/v)
Buffer EB (elution buffer)	1.25 M NaCl 50 mM Tris·Cl, pH 8.5 15% isopropanol (v/v)
PE	10 mM Tris·Cl, pH 8.0 1 mM EDTA