ISOLATION AND CHARACTERIZATION OF CALCIFYING BACTERIA FROM STEEL SLAG AND THEIR ROLE IN BIOMINERALIZATION

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IN
BIOTECHNOLOGY

Under the guidance of

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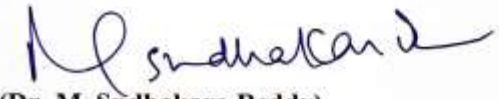
CERTIFICATE

Certified that the thesis "**ISOLATION AND CHARACTERIZATION OF CALCIFYING BACTERIA FROM STEEL SLAG AND THEIR ROLE IN BIOMINERALIZATION**" which is submitted by Miss Isha Rani, in the fulfilment of the requirement for the award of the degree of master of science in biotechnology in the department of biotechnology (DBT), Thapar Institute of Engineering and Technology, Patiala is a record of the candidate's own independent and original research work carried out by her under our guidance and supervision. The matter embodied in this thesis has not been submitted in part or full to any other Institute or University for the award of any degree.



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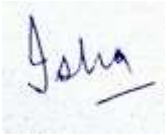
DECLARATION

I hereby declare that the work which is being presented in the thesis "**ISOLATION AND CHARACTERIZATION OF CALCIFYING BACTERIA FROM STEEL SLAG AND THEIR ROLE IN BIOMINERALIZATION"** submitted by me for the award of the degree of Master of Science in the department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab is true and original record of my own independent and original research work carried out under the supervision of **Prof. Dr. M. Sudhakara Reddy**, Professor, Department of Biotechnology. The matter embodied in this thesis has not been submitted in part or full to any other institute or university for the award of any degree in India or Abroad.



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ABSTRACT

Due to increased human activities and natural weathering processes like earthquakes, deterioration of construction materials has been occurring at increased rate. To sustain materials used in construction and to increase the service life of these materials, an optimum biotechnological method MICP has been exploited which causes production of microbial mortar. This method can be presented in a promising way to enhance the strength properties of cementitious materials. The objective of present work was to exploit the use of bacterial strain *Bacillus megaterium* or *Staphylococcus pasteurii*, which are isolated from alkaline environment of byproduct steel slag, in studying the enhancement of strength of mortar materials. Microbial process effectively reduced the water absorption levels i.e., less penetration of water occurred inside mortar specimens while increased compressive strength was also observed for the specimens with lower replacement levels. The present work mainly demonstrates the production of microbial mortar by ureolytic alkaliphilic bacteria and its utilization in strength enhancement of cement slag bacterial treated specimens. Partial replacement of cement with slag at lower levels can be taken into account on the basis of observed water absorption and strength properties and more studies can be further carried out for the same.

ABBREVIATIONS

Abbreviation	Word (s)
DNA	Deoxyribonucleic acid
EDTA	Ethylene Diamine Tetra Acetic acid
MICP	Microbially Induced Calcite Precipitation
MQ	Mili Q
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RPM	Revolutions Per Minute
SEM	Scanning Electron Microscopy
EDX	Energy Dispersive X-Ray Analysis
UAB	Ureolytic Alkaline Bacteria

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1. General Introduction

In the era of developing science and technology, mainly in field of civil engineering, where millions of buildings, houses, factories and industries have been continuously built on large scale, it requires the safety and durability of the same so that the constructed infrastructure persists for many more years. Although, structural designing in the previous decades was not much impactful but it was strong and resistant. However, in today's construction long term performance gets affected after getting exposed to aggressive natural or man-made environments. Its properties get depreciated and it becomes environmental concern. Thus, durability has become an important issue in construction of buildings and serious measures have to be taken to cure the problem.

Now-a-days, conventionally used construction materials are very much expensive, vulnerable or are causing harm to the environment in one or other way and due to this, it is more important to utilize economical and durable construction materials. Durability of any construction depends upon the types of materials used in it. Replacement of anciently used construction materials like mud, bricks, rare stones, lime etc. with todays' innovative materials like rice husk ash concrete, plastic bricks, waste from different kinds of mining industries like steel slag, fly ash etc. can cause noticeable durability enhancements and can prove better for construction industry.

Mortar is tough, durable and more inexpensive and thus is most widely used as a construction material. Besides having different potentials of concrete, some drawbacks have also been considered like less ductility, tensile strength or less resistant to deterioration and cracks. Deterioration may occur due to different man made or natural calamities and measures have to be taken to make the mortar more durable and resistant so as to maintain its quality and form till its best used time.

Strength issues in mortar can occur due to various native-born processes like earthquake, temperature and moisture changes, weathering of rocks and several other mechanisms like shrinkage, freeze-thaw reactions, compressive or some mechanical processes. The basic

reason for retrogression can be allocated to microscopically porous texture of mortar. Imbibition or diffusion of moisture in mortar members leads to corrosion which further leads to volumetric expansion problems and spall the concrete cover. Different remediating agents, waterproofing or surface treatments, like epoxy resins, can be applied to enhance durability of mortar but, mostly come with drawbacks like are expensive and also susceptible to UV radiations. So, there is a need for usage of a different strategy to make existing constructions more resistant and durable for long periods of time. Several research groups came out with a novel technique of using microbes to heal structures by say, biomineralization. It will undergo precipitation of calcium carbonate inside the pores of mortar and causes filling of pores and thus ingression of outside agents and permeability of gases is resisted and it becomes more substantial. This microbial based bio-sealant has introduced profound concept to the concrete industry for the enhancement of mortar performance.

Bio-minerals are ubiquitously present in the form of ant hills, beautiful corals, teeth, bones or rock etc., reported by Dhami et al (2013). These undergo precipitation by creating alkaline environment and produce minerals like calcite. Minerals produced by bacteria are called bio minerals while the process is called Biomineralization.

Calcium carbonate or $CaCO_3$ embraces about 4% of the earth's crust. It can be naturally produced by sedimentation of shells of snails, corals and shellfishes over millions of years or can exist in chalk, marble or limestone form or can be artificially produced by calcifying bacteria. It is one such mineral which is produced by ureolytic calcifying bacteria and contributes for strengthening of cementitious materials. Different kinds of polymorphs, of numerous shapes and sizes, are being produced, morphology of which is affected by different strains and species of bacteria.

These bacterial species include bacillus strains, mostly alkaline bacteria, which can survive high pH conditions as of mortar and thus can be used for calcium carbonate precipitation. Microbially induced calcium carbonate precipitation (MICP) is particular microbial plugging process or naturally occurring biological process, in which calcium carbonate precipitation is promoted, by microbial metabolic processes, in the form of calcite.

Aforementioned, steel slag, a by-product or waste from iron and steel making industry, is now-a-days, been actively studied for maximum utilisation. Also, utilizing steel slag for civil engineering purposes will lead to less usage of natural resources and their disposal can be reduced conveniently. This industrial waste, besides of having high alkaline pH, lime content

and other heavy metal content, can be used to isolate various microbial species, which will further be able to survive and easily adapt in high alkaline conditions of cement or mortar. Bacterial species, isolated from this industrial area have calcium carbonate precipitation ability and ureolytic activity and will easily adapt the environment of mortar which will help in increasing the durability of construction materials.

Also, steel slag can be used as a potential mineral admixture in mortar because it has cementitious properties which make it efficient for its utilization for the development of sustainable mortar design and eco-friendly environment. Cement can be potentially replaced, in different ratios, with steel slag in mortar and ultimately enhancement in strength can be achieved. This study was aimed to evaluate potential of ureolytic calcifying bacteria isolated from steel slag in enhancing durability of mortar and also using steel slag as a cement replacement in mortar to make it more sustainable.

1.2. Problems and Gaps in Studies

The today's building materials are not enduring enough and more of methods have to be established to make them life-long. One of the possible methods could be use of microbes. Microbial calcium carbonate precipitation is a process to produce bio minerals. It works on the principle of utilizing urea as a source of energy and producing ammonia which will create alkaline environment due to pH increase and ultimately causing Ca^{2+} and CO_3^{2-} to precipitate and form $CaCO_3$. Different kind of bacterial species like *Bacillus sphaericus*, *Pseudomonas aeroginosa* as reported by Achal et al (2011) are known to undergo bio minerals production and enhancement of the durability of concrete structures.

Other than the existing bacterial strains isolated from various environments and known to cause biomineralization, researchers are trying to isolate more of microbes with enhanced capability of the same. Other than isolating bacteria from soil, as done in previous researches, now researchers are trying to isolate and identify microbes from alkaline environment. Also, the existing construction material can be replaced with some economical and compatible natural resources which can fulfil the same purpose. Reason behind exploiting bacterial species to exhibit carbonate plugging in mortar was that microbes should have the capability to persist in the environment and this can be achieved by isolating microbes from same environmental conditions i.e., having high pH and reason behind using other natural resources as replacement is to be economical, eco-friendly and improving strength. So,

various industrial sites or mining areas have been seen to have high pH environment, thus steel slag, an industrial by product and waste is commercially exploited in present study to isolate different bacteria and as a replacement source with cement in mortar which will have applications in microbial mortar and also to enhance strength of mortar.

But various kinds of studies have to be done before exploiting any existing natural resource. As per the previous studies done on this industrial by product, it is dumped in yards in large amounts but now-a-days, it is commercially utilized as asphalt concrete aggregate, in CO_2 sequestration, aggregate in road and fertilizer in agriculture etc.. It showed some drawbacks also. Some properties of slag containing CaO and MgO content so, using it as replacement requires it to be treated and studying properties for its effective use. Because of high calcium content in steel slag, it reacts with CO_2 to form calcium carbonate which causes high alkaline pH and ultimately leads to volumetric expansion problems making it incompatible to be used as a replacement of cement in mortar. This problem can be resolved by using some techniques like heating over the slag for a period of time to let it expand completely but it is time consuming and inconvenient. So, a novel method of using microbes was being exploited so as to treat CaO content of steel slag and ultimately enhancing its strength.

1.3. Aim and objectives

The aim of this present study is to isolate ureolytic calcifying bacteria from an industrial by-product, steel slag, so as to exploit its use for enhancement of strength relating properties in mortar materials.

Objectives of this research include:

- Isolation and characterization of ureolytic calcifying bacteria for its utilization as $CaCO_3$ precipitating agent.
- Exploiting industrial by-product, steel slag as replacement of cement for evaluation of strength enhancement of mortar specimens.

CHAPTER: 2

REVIEW OF LITERATURE

Nowadays, reduction in energy consumptions is the most important challenge of future buildings i.e., from construction to destruction as reported by Asdrubali et al (2015). Industrial by products are the artificially produced materials produced as a result of industrial activities as mentioned by Kuntikana et al (2017). Major problems from these materials arise due to their inherent properties like alkalinity and high leachable metals content. Different kind of industries like iron and steel making industries and mining sites etc. caused production of large amounts of industrial by products and wastes, which are substantially generated all over the world, enforced challenges related with storage, reuse, transport and utilization. Increased awareness of humans towards global warming has led to increased concern over impact of anthropogenic carbon emissions from these industrial by products on the global climate as well as explained by Huntzinger et al (2009).

As reported by Doifode et al (2015), all the waste generated worldwide, municipal solid wastes constitute 14-20%, other wastes like construction and destruction waste 30% and 23% of mining and quarrying waste. These common waste sources include different kinds of manufacturing and production industries and also iron and steel making industries. These industrial wastes or alkaline residues (waste produced in alkaline environment) were produced each year globally with an estimate of 90 billion tonnes of production as investigated by Gomes et al (2016).

Alkaline residues are the wastes produced by industries like steel production and coal-fired power generation. It can be of different types, can be produced in nature in different amounts and also vary in their primary sources of alkalinity. Some of the main alkaline residues reported, include bauxite residues (constitute iron oxides, alumina-silicates, sodium carbonates and calcites) by Grafe et al (2011), Gomes et al (2016), concrete crusher fines (with quartz, calcite and albite in composition) by somasundaram et al (2014), Gomes et al (2016), chromite ore processing residues with free lime, portlandite, calcite and hydro-calumite present as dominant mineral matrix by Gomes et al (2016), Geelhoed et al (2003), steel works slag and fly ash (coal combustion) etc. Among the broad class of waste materials, the construction and demolition wastes (C&D waste) represent one of the largest constituents

of solid waste and among them, mixtures, concrete, asphalt and soil are present in various compositions as investigated by somasundaram et al (2015).

The materials falling into the category of construction and demolition (C&D) waste, including concrete crusher fines, steel slags, fly ashes, combustion residues etc. and these seem to have many potentials and environmental failures. Utilization of these kinds of wastes have been reported to reach over 80% in Netherlands, Denmark, and Germany regions as reported by Butera et al (2014) and Pan et al (2012) and the potential of these raw materials in CO_2 sequestration, as they are rich in different metal oxides like aluminium, iron and calcium etc. and thus caused accelerated carbonation. A common custom of implementation of concrete and other mineral residues as filler material for construction of road, embankments etc. was reported by butera et al (2014).

Besides potentials, several failures have been shown by these materials which are reported in various publications like steel slag weathering have been reported to cause increase in pH which ultimately causes surface and ground water pollution, reported by Mayes et al (2008), fly ash shows environmental concern from possible leaching of metallic compounds into nearby ground water or its direct inhalation may cause health hazards as reported by Kumar et al (2006), huge amount of unattended and unused industrial by products face problems relating safe dumping, explained by Kuntikana et al (2017).

Other than various kinds of metallurgical or mining industries producing huge amounts of industrial alkaline residues and debris, iron and steel industries are highly exhaustive in both materials and energy and steel production occurs at very large amounts. China is considered as the world's largest steel producer in the world as per world's largest steel producer, statistic - statista (2018).

2.1. Steel Production

It is one of the most common metal alloys and most substantially used materials in construction, ship building and infrastructure development. Among the world's largest crude steel producers, Tata steels of India is ranked at 10^{th} position with annual production of 25.11 million metric tons as per the world's largest crude steel producers in (2017) statistic - statista.

The world crude steel production ranges up to 1691 million metric tons in 2017 as per the statista portal which also depicted the crude steel production in India which was amounted to be approximately 90 million metric tons in volume in 2016. The annual growth rate of production was at approximately 10.7 percent in 2017 as mentioned in world crude steel production (2017) statistic - statista. As the steel is produced in India in this much large amounts, it also causes output of different kinds of large amounts of industrial by products and also alkaline residues.

Out of the two main ways of steel production industries i.e., iron-ore based steel making and scrap based steel production, the by-product mainly produced is slag (90% by mass) along with some amounts of dust and sludge and process gases, as reported in world steel association, steel industry by products. Also, depending upon the type of furnaces i.e., blast oxygen furnace, electric arc furnace and ladle furnace, used for steel production, different by-products exhibit different properties, as mentioned by Shi (2004).

Besides this much production of steel occurs in different nations of the world, huge amount of steel by-products and steel itself is an environmental havoc.

As steel is the most recyclable, innovative and sustainable material in 21st century as depicted by world steel association but different manufacturing and processing industries, including steel industries include various processes like sintering, coke making, iron making and production of final products like slabs and coils etc. It was reported that iron and other heavy metals processing causes dust emission with persistent polluting consequences in some regions. Also, steelworks units have been known to cause major emissions of heavy metals like zinc, copper, chromium, iron, nickel, arsenic etc. mentioned by Passant et al (2002) and Taiwo et al (2014), previous work by Moreno et al (2004) has identified steelworks to be a prime contributor to local particulate matter concentration. One steel plant containing 5 blast furnaces, on an average, causes 20 million tonnes of CO_2 emissions per year, as mentioned by Gielen D (2003). Production of large amount of scrap from iron and steel processing occupies larger land to dump and causes landfills, soil degradation by disposal of mine tailings cause solid acidification and deposition of trace elements which ultimately lead to environmental pollution.

Extraction of ore from the mining areas includes material that consists largely of waste and less of valuable portion of crude ore. The waste thus generated gets categorized into four kinds: mine waste, mine water, dump heap leach and tailings etc. which is considered as

environmental havoc as these cover hundreds of hectares of lands, causes metallic leaching. Huge amounts of waste waters are also produced in smelting process causing land and water contamination. Also, hazardous waste including process waste and refining debris is generated as reported by Dudka et al (1997).

2.2. Steel Slag

It is a by-product produced from iron and steel ores by means of metallurgical process as explained by Piatak et al (2015). Slag production from pig iron and steel depends upon types of furnace used at the time of refining of metal, and also composition of raw materials. One of the wastes produced during processing of iron and steel ore into final products is steel slag. It is basically produced from either conversion in basic oxygen furnace from iron to steel or steel making in electric arc furnace by melting existing steel scrap to produce steel. The hot liquid metal from the blast furnace was charged to basic oxygen furnace along with scrap and fluxes (lime and dolomitic lime) and provided with high-pressure oxygen. This reaction with oxygen helped in removing impurities which consisted of carbon as carbon monoxide, silicon, phosphorous and manganese etc. These elements then react with fluxes to form steel slag as published by Shi (2004).

In case of electric arc furnace, cold existing steel scraps are used instead of hot metal then it is provided with iron scraps and pig iron. An arc is formed by supplying electric current through electrodes which generates enough energy to melt the additional scrap. After getting required composition of steel by adding additional metals, oxygen is also added to get purified steel. Slag is then obtained floating as upper layer above molten steel which is tilted to pour off.

On the basis of types of steel used, steel slag can also be classified into carbon steel slag and stainless steel slag as reported by Huang et al (2012).

Current total productions of steel slag in India, are around 12 million tonnes per annum as mentioned in Indian Minerals Yearbook (May 2016), which is far behind the developed countries. Blast furnace slag production ranges from 300 to 500 kg per tonne of crude iron obtained. During the year 2016-2017, Tata steel plant reported to produce 3,783,844 tonnes of blast furnace slag as reported by Indian minerals yearbook (2017).

This by-product steel slag comprises of several chemical, mineralogical and cementitious properties.

2.2.1. Physical and chemical characteristics:

Steel slag has a density of about 3.3-3.6 g/cm^3. The presence of Fe content makes it appear hard and wear- resistant. It has grindability index of 0.7 as compared to blast furnace slag with 0.96 and standard sand with 1.0 grindability index. The chemical composition of steel slag varies with grade of steel, type of furnace used and its pre-treatment method. Table (2.1) depicts the chemical content of both blast furnace and electric furnace types of slag.

Table 2.1: Chemical composition of steel slag (source: Huang et al, 2012)

Oxides%	CaO	SiO_2	MgO	FeO	Fe_2O_3	Al_2O_3	MnO	P_2O_5
BOFS	45-60	10-15	3-13	7-20	3-9	1-5	2-6	1-4
EAFS	30-50	11-20	8-13	8-22	5-6	10-18	5-10	2-5

Main mineral phases reported to be present in steel slag are free lime (CaO), dicalcium silicate (C_2S), tricalcium silicate (C_3S), tetra-calcium alumino ferrite (C_4AF), RO phase (CaO-FeO-MnO-MgO solid solution), merwinite and olivine etc. as reported by Kourounis et al (2007).

Table 2.2: Characteristics of steel slag and its applications (source: Huang et al 2012)

Characteristics	Applications
Highly alkaline and porous	Waste water treatment
Fe, FeO_x components	Reclamation of Iron
RO phase components	Fluxing agent
MgO, CaO components	CO_2 sequestration
FeO, SiO_2, CaO components	Cement clinker raw material
Fertilizer components	Improvement of soil and fertilizers
Cementitious components	Cement and concrete manufacture
Coarse texture, adhesive, wear-resistant	Aggregate in road construction

2.2.2. An Environmental concern

Process of separation of metals from ore causes production of large amounts of steel slag which is reported to be extensively dumped at smelting sites and thus causing land pollution as mentioned by Piatak et al (2014). One of the studies by Piatak et al (2010) indicated that some toxic elements like Zn, Pb, As, Ba, Cd, and Cu etc. were present in high concentrations. Large concentrations of Cr, Pb and Zn were found in ground water exactly underneath steel slag deposits by Bayless et al (1998). Also, usage of steel slag is shown as filler in the construction industries, mentioned by Shen et al (2009), Ahmedzade et al (2009). It is also reported by Yildrim et al (2009) that due to very crystalline structure of steel slag, it shows very weak cementitious properties. A great environmental concern arises due to leachate generation from high amount of dumping of steel slag as described by Tiwari et al (2015). Utilization of steel slag in agricultural purposes has been seen to affect soil properties of plant by Humaria et al (2014) and Chand et al (2015). Harmful effects of steel slag have been observed by IMY (2012) upon aquatic environment due to its irrational dumping. In comparison to blast furnace slag, the CaO and MgO content of steel slag can cause volumetric expansion if not appropriately tested before using as construction material as reported by Wang (2011).

This much amount of mining, smelting and processing is not much economical in present world. Dumping of existing by products, recently produced debris, dealing with environmental impacts and processing and transportation also requires huge capital resources. Hence, steel slag which is a by-product of steel industries can potentially turn from by product to an environmental hazard, thus needed to be handled with care.

2.2.3. Potentials of steel slag

Besides, steel slag has many environmental concerns; it can be potentially used in various industrial applications. Modern construction industries consider steel slag to be a renowned and helpful raw material for high scale constructions. There is approximately 10% of waste steel content in steel slag which can be retrieved through, sorting and screening etc. as explained by Huang et al (2012). As steel slag contains high CaO content, if it is present in more than 50% amount it can be used as a fluxing agent for sinter ores. Also, addition of slag can cause uplift in quality and decrease in cost of sinter ores. Durability, high strength, high bulk density and rough texture of steel slag makes it compatible to be used in hydraulic

engineering purposes and it can be processed to be used as aggregates of high quality as published by Huang et al (2012). An experimental investigation done by Yan et al (2000) demonstrated that besides Ca-, Si- and Mg- containing minerals are important but highest acid neutralization capacity is provided by the Calcium content and concluded that acid neutralizing capacity depend upon different pH levels of steel slag, their mineralogical properties and on the reaction time.

Proctor et al (2002) have reported the stability of steel industrial slag to be more than other aggregates and brilliant adhesion properties have been shown in asphalt and concrete. It can also be placed in and near water bodies for bank stabilization and perform duties as an aggregate for drainage trenches. Significant portion of slag, left after recycling to blast furnace, have been investigated to be used in road construction due to its excessive stability and wear resistance as reported by Chaurand et al (2006), Alexandre et al (1993).

Steel slag can also be used in manufacturing of paints and in brick making as published by Pajgade et al (2013). Experts have agreed to the metal stabilizing potential of steel slag in contaminated soils. The slag has also been directed by PWD to be used as a replacement to river sand and is comparatively cheaper also as investigated by Rao et al (2014). Dhoble et al (2012) reported that the effective properties and characteristics of steel slag effect its civil engineering construction applications.

To potentially utilize the cementitious characteristics of steel slag, it can be used as an aggregate in concrete by exploiting its properties in cement industry.

As environment is getting deteriorated and concrete industry is evolving, legislations and commercial trends restricted the exploitation of natural resources and instructed concrete industry to reduce CO_2 emissions thereby minimizing environmental hazards. Also, there is an increased demand of raw materials and sustainability of natural resources is decreasing day-by-day, it is expected to use alternative raw materials which also proved to be economical. CO_2 emissions due to fossil fuels and industrial purposes amount to be around 2.5 billion metric tons in the year 2015. CO_2 emission not only occurs due to major use of coal in cement production but also occurs due to clinker production.

2.3. Building materials: Cement and Concrete

The most widely used construction material is concrete. In many countries ratio of consumption of concrete to steel exceeds ten to one as reported by Mehta et al (2001). Besides, concrete is neither as tough nor as strong as steel, still it is used most widely in construction because of numerous numbers of reasons. First is its magnificent resistance towards water as comparable to wood. It can withstand against deterioration caused due to water which makes it ideal material for building water storage structures etc. Also pavements, floors, piles, foundations have been seen to be made using concrete. Second, concrete reinforced with steel bars provides excellent resisting forces. Third, it has excellent plastic consistency when freshly made and can be used as a material to flow into prefabricated formwork which can be removed later. Fourth, it is the cheapest and voluntarily available material for construction engineers.

Concrete, a composite material, comprises mainly of mixture of inert mineral aggregates like sand, crushed stone, gravel and cement i.e. binding medium which are embedded particles of aggregates should be essentially contained. An aggregate is any granular material such as sand which can be used as a cementing medium. It can be classified as coarse and fine based upon sizes of particles larger or smaller than 4.75 mm respectively as reported in Mehta et al (2001).

The production of concrete for buildings and infrastructure require most important raw material i.e., cement. Global production of cement is likely to be increased from 3.27 billion metric tons in 2010 to 4.83 billion metric tons till 230. India is the world's second largest producer of cement in the world and in the year 2017, India undergoes an average of 280 million metric tons of cement production as depicted by Cement production in India, statistic-statista (2017)

Cement is the most widely used construction material and is used to bind building materials together and in production of concrete. Its consumption and manufacture is closely related to building activity and thus to the general economic activity as reported by Hendricks et al (2004).

It is widely produced due to relatively more economical and highly denser which limits ground transportation because of high cost. It can be distinguished into hydraulic and non-

hydraulic cements. The former constitute oxides and silicates that causes absolute setting and hardening even after exposure to water as reported by The Statistic Portal - Statista (2017).

Beyond production of cement, its cost, effects and consequences, future buildings are suspected to most important challenge i.e., reduction of energy consumptions in all phases, from construction to demolition. These roughly consume about 40% of world global energy as per united nation environment program by Asdrubali et al (2015). This energy generation requires huge amount of usage of water causing water scarcity also. Huge amount of carbon-di-oxide emissions in the atmosphere mainly occurs due to a) deforestation and other land degradation b) oxidation of fossil-fuels and due to c) carbonate decomposition. Cement production involves decomposition of carbonates and thus emissions of CO2 in highest amount by Andrew (2017).

In case of cement production, CO_2 emissions occur either due to the decomposition of carbonates into oxides by providing heat or due to fossil fuels combustion to produce energy to heat the raw materials as reported by Andrew (2017). According to recent studies done by IEA (2016), cement industry contributes to about 8% of total CO_2 global emissions and these industrial processes or energy emissions were often depicted separately in global emissions inventories by IPCC (2006). Several researches have been conducted to reduce CO_2 emissions by using alternative binders and replacement of cement with other potential recycled materials like glass by Castro et al (2013), basalt aggregates by Ingrao et al (2014), ceramic or other aggregates. Carbon capture and storage technologies (CCS) implementation into cement production can provide more remarkable carbon footprint reduction as reported by CemWeek magazine (2013). But an increase in cement manufacturing due to current construction technologies and ever increasing population demands an increased utilization of large amount of energy which will become an issue of more environmental concern. Clinker production in cement manufacture also involves high capital investment as reported by Aruntas et al (2010).

Alternatively, investigations have been done for cement manufacture with an additive instead of using existing depleting natural resources. This will also prove more economical and feasible method as reported by Aruntas et al (2010) and tirelessly increasing industrial waste amounts due to this industrialization and urbanization have created a critical issue whose management is further raising critical concern as concerned by kim et al (2018). The

extensively growing greenhouse gas emissions and increased global demand have motivated people to find alternative for the management of the same.

Hence, there is a need to utilize some alternative pozzolans in cement manufacture to decrease unhealthy emissions and improve energy efficiency. This challenge of reduction in environmental footprint and simultaneously dealing with ever-increasing demand of this essential material has to be achieved. Utilization of clinker replacements from minerals processing or power production reported by Ishak et al (2015) is done for better process integration. There are also studies relating use of fuel switching and also some alternative fuels as by Rahman et al (2015). Recent study by Singh et al (2018) deals with dumping of waste from agricultural area by using them as coarse aggregate in concrete manufacture.

2.4. Industrial by-products: as replacement source

Use of alkali-activated cement (AAC) as substitutive cementitious material along with alkali activator makes them potential to become a cementing character and show high mechanical performance. Alkali activated materials are natural or man-made aluminosilicates which get activated using alkaline carbonates, hydroxides, silicates etc. as described by Puertas et al (2018).

These substitutive materials can act as industrial by-products and are produced in various construction and mining industries. Industrial by-products are the artificial materials produced as a result of different mining and industrial activities. Extremely large amounts of dumped unused by-products could be used for their sustainable utilization instead of becoming environmental hazard as reported by Kuntikana et al (2017). Studies have shown the usage of these materials as aggregates and pozzolans etc. in the production of fertilizers, landfills at mining areas, bricks and pavements manufacture and flue gas desulfurization etc. as by Yao et al (2015), Ahmaruzzaman et al (2010).

Bulk usage of industrial by products may impose challenges like high alkalinity, leaching of heavy metal content or presence of trace amounts of radioactive compounds. Hence, it is important to isolate industrial by-products to diminish their ill effects and make them wholly or partially stable. A conclusion is given to this issue to establish synergy between various industries so that they can utilize each other's waste and help in reducing detrimental effects on environment. But this approach needs that synthesization of various aspects of the same should be ascertained as suggested by kumar et al (2006).

These industrial wastes can be used as supplementary cementitious materials (SCMs). They exhibit cementing properties and may include fly ash, silica fume, and steel slag as reported by Crossin (2015). Industrial by-product having potential for replacement have been reported in various studies. As reported by Kim et al (2017), slags (industrially generated wastes) show potential pozzolanic properties and its use as a concrete constituent, either by using it as a replacement of aggregate or use as cementing material or both, is also demonstrated.

Production of fly ash occurs by coal combustion for the generation of electricity and is captured by electrostatic precipitators. These can be calcareous or siliceous depending upon the type of coal used. It consists of unburn carbon and crystalline matter as reported by Gomes et al (2015).

2.4.1. Fly-ash (FA)

In case of getting a desired characteristic of concrete, different kinds of adjuvants have to be added to strengthen construction materials. To obtain strong and durable mortar with proper setting time, mixing and compaction, coal burned industrial by-product of the coal power plant i.e., fly ash is being used to as a partial replacement along with rice husk ash and steel fibres in concrete. It has also been reported to show similar characteristics as of natural pozzolans by Lakra et al (2016).

Heidrich et al (2013) depicted the composition of fly ash generated after coal burning. It mainly consisted of 75-85% of iron oxides, aluminium and silicon, lime content was seen to be less than 10% in siliceous ashes while more was present in calcareous ashes. Table (2.3) summarizes the whole chemical composition of cement and coal fly ash depicted by Rafieizonooz et al (2016).

Table 2.3: Comparative study of composition of cement and industrial by-product coal fly-ash

Material	**CaO**	**SiO_2**	**MgO**	**Na_2O**	**Fe_2O_3**	**Al_2O_3**	**MnO**	**P_2O_5**	**K_2O**	**SO_3**
Cement	62.39	20.4	1.55	-	4.19	5.20	-	-	0.005	2.11
Coal fly ash	10.7	47.6	150	0.26	7.42	23.8	0.120	0.16	1.68	0.75

Supit et al (2015) investigated the compressive strength and durability tests like chloride permeability, water sorptivity and porosity by preparing series of concrete mixes containing 40% and 60% cement replacement by fly ash. After 3d, 7d, 28d, 56d and 90 days, reduction

in chloride penetration was seen more in 40% replaced specimens than in 60% specimens and a subsequent increase in compressive strength was also observed.

Fly ash of specific gravity 2.16 was used for making specimens of 10%, 20%, 30%, 40%, 50% and 60% replacement of cement. 20% replacement with fly ash showed improved mechanical properties. Compressive strength was noted to be decreased with increasing level of replacement as stated by Murali et al (2012). Also, increase in compressive strength and bond strength was noted with increase in curing time.

Fly ash with 2 different fineness properties was used to prepare mortar specimens. A cement replacement of 15%, 30%, 45% and 60% with fly ash was done. Test results for setting time demonstrated that finer fly ashes of 60% and 90% fineness at similar replacement level of fly ash inferred reduced setting time as compared to 40 F fly ash cement mixes. Choi et al (2011) concluded that setting time increases with increased fly ash percentage. Also, 15% and 30% replaced cement mixes showed faster setting time as compared to control mixes. Compressive strength of concrete was shown to be reduced with increasing fly ash replacement ratio.

2.4.2. Rice-husk Ash (RHA)

Rice plant, which undergoes silica absorption from the soil, has an outer covering called rice husk. It produces 80%-85% concentration of silica after burning. Husk mainly consisted of 25%-30% lignin, 50% cellulose and almost 20% silica. As reported by Ye et al (2018), almost 200 kg of ash was being produced on complete combustion of one ton of rice husk and was called rice husk ash (RHA). It could be present in 2 forms i.e., amorphous RHA which was highly reactive and a potential pozzolan for cement replacement and the crystalline RHA as reported by Sata et al (2007).

Rice husk ash (RHA) is one of the main residues produced in agricultural industry and its use as supplementary cementitious material in concrete in sustainable environment solution. Its usage can not only decrease the fuel demands in cement manufacture but is eco-friendly and can improve the durability of cement concrete and mechanical properties as mentioned by Balapour et al (2018).

It was concluded by Balapour et al (2018) that 2.5% of nano-rice husk ash replacement with cement showed higher compressive strength as compared to control mix after curing at 3 days

while the one with 7.5% replacement exhibited reduced compressive strength. An increase in replacement level in concrete mixes also showed higher chloride resistivity.

Concrete mix proportions with 10, 15 and 20% partial replacements with rice husk ash were observed for compressive strengths and split tensile strengths up to 56 days. Compressive strength was observed to be increased up to 15% replacement and decreases after that. It is believed to be increased due to pozzolanic properties of RHA. The porosity of the specimens is believed to be decreased with increased curing time as explained by Chopra et al (2015).

A partial replacement percentage from 5% to 25% of rice husk ash was also observed by Ahmed et al (2018) which showed decrease in compacting factor value or workability and cause an increase demand for water. Bulk densities are also believed to be decreased with increased replacement percentage. Also, similar decrease was observed in case of compressive strengths.

Comparable results were observed by Juma et al (2012) and concluded that compressive strengths had relatively increased due to replacement of concrete with rice husk ash.

2.4.3. Ground-granulated blast furnace slag (GGBS)

Ground-granulated blast furnace slag (GGBS) is used as a mineral admixture so that it can increase durability and decrease porosity. It is mainly a by-product produced in the manufacture process of pig iron which consists of alumina, lime and silica that make up cement. The molten slag produced after process in blast furnace is rapidly chilled to make fine-aggregate-sized-particles and predominantly contain glass particles in it. It was concluded that the resultant product GGBS is obtained by fine grinding of the end product.

It is clear from the experimentation by Nili et al (2015) that compressive strength observed after GGBS replacement was significantly increased as compared to normal concrete mixes. Replacement was done at 20%, 30%, 35%, 50% and 65% levels. A 2.8% increase in compressive strength has been observed in specimens with 20% replacement as compared to control mixes after 91 days curing. Furthermore, no desirable results have been obtained after a replacement level of 35%.

Influence of GGBFS replacement with Portland cement up to 60% has been done under an experiment conducted by Ozbay et al (2016). An enhanced workability and beneficial effects has been observed till 20% replacement. Also, increase in replacement percentage caused

prolongation of setting times of concrete. Increase in setting time by factor of 2.75 and 2.25 had been observed at replacement level of 60%. At 90 days curing, monitoring of wet cured concrete showed positive increase in compressive strength when replaced with GGBS at 50% and 60% substitution.

2.5. Steel slag in concrete

Steel slag usage as replacement of natural aggregate in concrete was initially started due to its high availability in terms of natural resources and high characteristic properties. These physical properties of steel slag make its potential to be used in cement and concrete structures and enhanced the overall properties of end products as depicted by Wang et al (2010).

Steel slag aggregates collected from various sources may vary in their physical and chemical properties. Iron and steel slag as examined by Rajan (2014) are non-metallic and lack any kind of hazardous content. As, different kinds of slags are being produced and studied, steel slag from steel making and refining industries is not extensively known as said by Tiwari et al (2016).

Figure 2.1: The basic process of generation of slag and its kinds (source: Tiwari et al, 2016).

As per Neeraja et al (2015), 300 to 540 kg of blast furnace slag per tonne of pig iron is produced, in general.

2.5.1. Composition and properties

Composition of steel slag also constituted some hydratable oxides like CaO and MgO along with silica, iron oxides and aluminium oxides etc. which caused volumetric instability of the resultant structures. These volume deformities caused due to high lime content lead to the possibilities of internal stress within the particles of mortar and ultimately caused damage as concerned by Palankar et al (2016). Problem can be resolved by treating the slag to

weathering process which will undergo several physical changes along with reducing the free lime content. These changes can be visibly seen in form of thin coat of calcite layer on the surface.

Due to the rough texture, better interlocking strength and angular shape of steel slag particles, studies by Taha et al (2014) had reported enhanced mechanical properties of ordinary cement concrete structures after using steel slag as a coarse aggregate. Also, some strength reduction behaviour has also been reported in studies done by Gonzalez-Ortega et al (2014) after incorporation of steel slag as aggregate in concrete.

2.5.2. Utilization as cement replacement

Besides having many environmental concerns and potentials, it has now being used in cement manufacturing as per latest trends. For the manufacture of one tonne of cement, it requires almost 1.5 tonnes of limestone and if out of that, 10% of limestone is replaced by blast furnace slag, it created a market for 60 million tonnes of Blast furnace slag in one year as reported by Tiwari et al (2016). This helps in almost complete utilization of blast furnace slag produced and subsequent emissions of CO_2 can be reduced. But production of cement required material having less iron content and this requirement matches with blast furnace slag containing less amount of iron as compared to steel slag depicted by IMY (May, 2015). Slag utilization in making of portland slag cement should be up to the Indian standard specifications as increased amounts can cause deleterious effects or decrease in compressive strength as reported by Tiwari et al (2016).

The hydration rate of Portland cement after incorporating steel slag has been studied by number of researchers. Wang et al (2010) studied the influence of steel slag on hydration of cement at the time of hydration of the complex binder and concluded that steel slag and cement do not react with each other's hydration products until environment is changed in early ages. However, in later ages, slag improves cement's hydration conditions and this effect increases with increase in slag replacement ratio.

Uneven distribution of RO phase of steel slag particles caused introduction of defects in concrete, if incorporated as fine or coarse aggregate as mentioned by Pang et al (2015). Thus, its utilization can result in unstable service structure of concrete. The angular shape particles causes reduced mobility of concrete which resulted in reduced workability. Also, steel slag aggregate showed more compressive strength as compared to normal aggregate with 17%

increased modulus of elasticity. Further, conclusion was drawn that increase in replacement ratios caused modulus of elasticity to increase more.

2.5.3. Utilization as coarse aggregate

Utilization of steel slag as a coarse aggregate is done to reduce the use of other conventional materials and leading to rapid slag utilization, a topic of great environmental concern. Poonkodi et al (2018) concluded that a 100% replaced steel slag concrete showed workability near to conventional concrete. Compressive strength was believed to increase with ageing period of steel slag aggregate. Steel slag showed a specific gravity of 13.24%, crushing value of 18.23% and impact value of 18.90% higher than that of conventional crushed stone coarse aggregate.

Utilization of steel slag has been accounted to show some environmental aspects also. It shows slower activity in terms of cementitious composition as compared to cement clinker because of its thermal history as explained by Hu et al, 2008. Use of superfine ground steel slag is being used to enquire about its pozzolanic activity as it is believed that pozzolanic activity increases if the material is subject to grinding as reported by Shi et al (2015).

Palankar et al (2016) reported the different replacement levels of steel slag coarse aggregate to evaluate strength and durability properties. At replacement ratios of 0%, 50% and 100%, early strength properties were significantly decreased while no later age strength reduction was observed. Incorporated concrete with steel slag aggregate showed higher water absorption while acid and sulphate resistance subsequently decreased.

2.6. Microbial treatment of concrete

As concrete is most widely consumed material in the world, concrete structures are high on load bearing capacity but have weak tensile strength which then requires steel reinforcement for support. But weathering conditions and various processes of concrete hardening causes cracks in concrete, as explained by Pappupreethi et al (2017), vulnerability of mortar to crack or get deteriorated is also explained by Kumar B. (2015) and ingression of water in between cracks ultimately causes deterioration. In order to enhance, remediate or restore range of building materials, modern technology or costly repairs can be done but we require a technique which can contribute to both better sustainability to the environment and mechanical and durability properties. One of the possible applications can be

Biomineralization process involving bacterially induced calcium carbonate precipitation which results from metabolic processes of some specific microorganisms.

2.6.1. Micro-organisms and their metabolism

Microbial enzymes have secured attentiveness for their large scale use in environment-friendly industries. Enzyme mediated processes have reduced process time and are non-toxic and cost-effective. Microbial enzymes are more active and stable than plant and animal enzymes. Different downstream processes can be used to extract enzymes which can be restricted physiologically and physio-chemically.

Bacteria are pervasive, can grow at different habitat; can have numerous morphologies, and roles for example, precipitation. They can be directly or indirectly involved in decomposition leading to growth and also chemical alterations as explained by Adams et al (2005). Bio-mineralization is the process of producing minerals by use of micro-organisms. Microbially induced calcium carbonate precipitation (MICCP) is one such process. Minerals thus produced may include different kind of polymorphs such as calcium carbonate polymorphs, silica, iron oxides etc. Bio-deposition may involve a range of microbes, pathways and environments. Calcium carbonate precipitation, from alkaline calcifying bacteria, is previously being carried out in range of natural environment. Bio-mineralization thus can be applied in remediation and restoration of building composites exploiting their technical applications in other fields as well.

Minerals concoction, mediated by prokaryotes, can occur in two ways: Biologically controlled mineralization and biologically induced mineralization. The former includes direct mineral synthesization on specific location on or within the cell while organisms' metabolic activity causes production of minerals in the latter.

There can be different ramifications associated with MICCP: carbon-di-oxide sequestration and dolomite precipitation, solid-phase capture of inorganic contaminants, formation of pathological mineral concretion.

2.6.2. Biomineralization

Biomineralization is of great significance in scientific and commercial applications and have great influence in fields of microbiology and geology etc. The process by which micro-organisms show activity which results in precipitation of minerals is Biomineralization. Mineral deposition can occur within or outside the surface of micro-organisms. Different kinds and number of minerals have been found to be produced using microbes which can vary in sizes and morphology as well such as amorphous, crystalline or some temporary forms.

Calcium, Magnesium, Phosphate (in vertebrates), silicates (in algae and diatoms) and carbonates (in invertebrates) minerals as mentioned by Dhami et al (2013) varies greatly on the basis of their dissolution conditions due to varying pH as explained by Adams et Al (2005).

Prokaryotic mineral synthesization is broadly classified into 2 different fundamental groups: Biologically controlled mineralization (BCM) and biologically induced mineralization (BIM). Although, an another kind of bacterially influenced mineralization has been reported and published by Anbu et al (2016) according to which organic matter of the cell surface causes passive mineral precipitation for example in case of extracellular polymeric substances associated with biofilms.

2.6.2.1. Biologically controlled mineralization:

In this, direct minerals synthesization occurs under specific conditions and at specific locations within or outside or at periphery of bacterial cell, most preferably intracellularly which provides confined space along with some proteins or lipids so that a stable matrix could be formed for cations condensation as explained by Dhami et al (2013). Nucleation and growth of minerals is also governed by micro-organisms as explained by Anbu et al (2016). The minerals of this kind of biomineralization are present with narrow size distribution with well-ordered structure, consistent and possess crystal habits, said frenkel et al (2003).

2.6.2.2. Biologically induced mineralization:

An interaction between biological activity and environment causes precipitation of minerals as a product which can be termed as BIM, said Sarayu et al (2014). These minerals that are formed, due to metabolic activity of microbes, generally nucleate and grow at extracellular

surface. Various, bacterial surfaces other than cell wall like polymeric substances (slimes, biofilms etc.) which are emitted by bacteria itself, act as site for nucleation and growth. Sometimes, due to secretion of more than 1 metabolic product, these result in subsequent deposition of chemically reacted minerals as explained by Frenkel et al (2003). Once synthesized, these bio-minerals can be altered by altering surrounding like pH, composition of its microenvironment or may be CO_2, thus complete activity depends upon existing conditions. It is, in essence, comparable to inorganic mineralization because the minerals possess crystallo-chemical features. Minerals are thus characterized by vast particle size distribution, lack of crystal morphologies, have poor crystallinity and poor mineral specificity as reported by Frenkel et al (2003).

2.6.3. Microbially Induced $CaCO_3$ Precipitation (MICCP)

A supersaturated solution formed due to microbial cells and their metabolic processes causes formation of calcium carbonate which refers to as microbially induced calcite precipitation. It is basically a bio-geo chemical process which causes precipitation of $CaCO_3$, which sticks the material particles together and ultimately increases the strength and firmness of the material, explained Mortensen et al (2011).

Calcium carbonate precipitation induced by bacteria have been widely studied for its potential uses in various sectors like construction materials, hydraulics, cementation of porous media etc. utilization of MICP for engineering purposes, specifically in construction is reviewed by some researchers. Role of MICP in improving and rehabilitating construction materials has been explored by Muyunk et al (2010). Also, siddique et al (2011) reported use of MICP in concrete in construction industry.

It is an effective and eco-friendly technology and it can be utilized to resolve various kinds of environmental problems, as discussed by Muyunk et al (2010). Ganendra et al (2014) found this process to be beneficial over ureolysis-driven process as it involves decreased threat of pollution. Applications of MICCP involving biomineralization have been reported in various fields like remediation of ground water contaminated with heavy metals, by Fujita et al (2004), historical stone monuments restoration, by Daskalakis et al (2015); Jroundi et al (2014) and improvement in durability of concrete structures, by Achal et al (2011); Bundur et al (2015) and Dhami et al (2013).

2.6.4. Microbial concrete

As mentioned by Joshi et al (2017), it is a microbial based strategy which involves treatment of mortar with microbial cultures so as to initiate calcium carbonate precipitation. The ability of different kinds of bacterial species like ureolytic bacteria, sulphate-reducing bacteria and uni-cellular cyanobacteria etc. has also been reported.

In case of microbially-induced calcium carbonate precipitation, chemical composition of bacteria, due to its cellular activity, is responsible for biomineralization as explained by Philips et al (2013). When the cells serve as nucleation sites for calcite precipitation, it can possibly undergo biologically influenced mineralization also. It is a kind of bacterial mineralization taking place outside the bacterial cells.

There are number of factors which can affect the Biomineralization process. Key factors mentioned by Dhami et al (2013), Sarayu et al (2014) are: a) dissolved inorganic carbon concentration in microenvironment b) pH of surrounding solution c) presence of appropriate nucleation sites d) availability of reactive calcium ions e) urease enzyme genetics f) calcium carbonate crystal polymorphs. The nucleation sites impact the critical saturation state (S_{crit}) which is the state of saturation for nucleation to take place as mentioned by Philips et al (2013).

Different hypothesis have been made regarding the factors responsible for bacterial Biomineralization. Three of them are mentioned by Dhami et al (2012) which are as follow: a) any kind of autotrophic or heterotrophic process can be involved in microbial metabolism producing bio-minerals as by-product like urea hydrolysis which causes increase in pH, shift in carbonate-bicarbonate equilibrium, produces more CO_3^- ions and precipitates $CaCO_3$ in presence of Ca^{+2} ions (most accepted method by deJong et al (2010), Muyunk et al (2010) b) carbonates nucleation occur on the bacterial cell wall, may be due to ion exchange across cell membrane or adsorption of ions by cell wall functional groups as studied by Castaneir et al (2000) and c) important role is played by extra-polymeric substances to trap calcium ions to influence precipitation.

Among all these processes, hydrolysis of urea is advantageous over other processes. This process is energy efficient and large amounts of carbonates can be precipitated in small time period said Muyunck et al (2012). Catalysis of urea occur in the presence of urease enzyme which causes hydrolysis of urea into ammonia and carbonic acid. Anbu et al (2016), Dhami

et al (2013) reported that during the activity of microbial urease, hydrolysis of one mole of urea intracellularly produces one mole of ammonia and one mole of carbonic acid which then spontaneously hydrolyses to form 1 mole of ammonia additionally along with one mole of carbonic acid as defined in equations below:

$$CO(NH_2)_2 + H_2O \xrightarrow{\text{Urease}} NH_2COOH + NH_3$$

$$NH_2COOH + H_2O \longrightarrow NH_3 + H_2CO_3$$

Then, equilibrium of these products occur in water to form bicarbonate ions, 1 mol of ammonium ions along with hydroxide ions which causes increase in pH of the surroundings which then shift the bicarbonate equilibrium and formation of carbonate ions occurs. More generation of ammonium ions increases more pH and reaction continues spontaneously to form more of calcium carbonate

$$H_2CO_3 \longrightarrow 2H^+ + 2CO_3^{-2}$$

$$NH_3 + H_2O \longrightarrow NH_4^+ + OH^-$$

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

$$Ca^{2+} + CO_3^{-2} \longrightarrow CaCO_3 \ (K_{SP} = 3.8 \times 10{-}9)$$

Calcium carbonate precipitation occurs at the surface of bacterial cell if sufficient amount of both Ca^{2+} and CO^{3-} ions are present in cell.

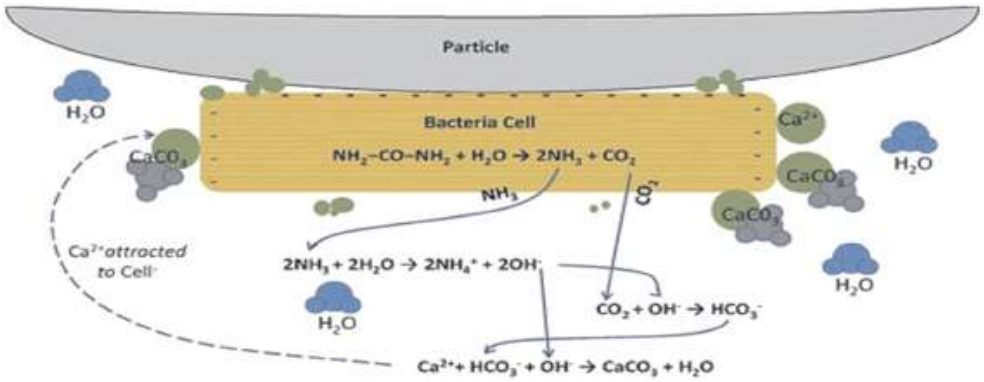

Figure 2.2: Nucleation site of bacteria for $CaCO_3$ precipitation in sand particles (Dhami et al 2013)

Although this activity of urease enzyme is widespread among various different groups of microorganisms, bacterial species of bacillus group are predominant in this activity, as

reported by Achal et al (2015). Mortensen et al (2011) reported that ureolytic bacteria possessing this enzyme are commonly available in subsurface.

2.6.5. Polymorphs of calcium carbonate

Different polymorphs or phases of calcium carbonate precipitated by the process of biomineralization have been reported by Anbu et al (2016); Dhami et al (2013); Rusznyak et al (2012) such as the anhydrous polymorphs like calcite, vaterite, aragonite; hydrated crystalline polymorphs like monohydrocalcite ($CaCO_3.H_2O$), ikaite ($CaCO_3.6H_2O$) and numerous number of amorphous phases which vary in degree of hydration. Out of these three anhydrous polymorphs, most commonly precipitated are the calcite and vaterite. Dhami et al (2013) reported that phase of polymorph, concentration and morphological characteristics of calcium carbonate depends on degree of supersaturation, pH, $[Ca^{2+}]$ / $[CO_3^{2-}]$ ratio and temperature. Precipitation of calcium carbonate occurs if saturation index goes above 1, where saturation index, SI = log Ω = log IAP/Ks. Here, Ω is saturation state of the system, IAP refers to ion activity product and Ks refer to thermodynamic solubility product of the phase.

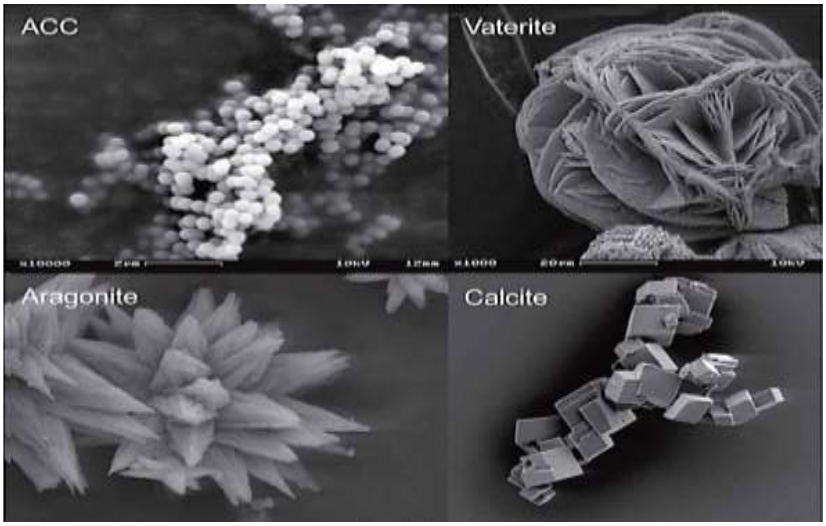

Figure 2.3: Different polymorphs of $CaCO_3$ (Source: Dhami et al 2013).

In addition to applications of MICP in various fields, many researchers have reported the use of MICP for the production of microbial concrete and for enhancement of mortar structures.

2.6.6. Utilization in strength enhancement

Microbial concrete have been reported to be used as alternative to surface treatment by Kumar (2015). Applicability of this novel bacterial based strategy has been investigated by khaliq et al (2016) in enhancing the compressive strength of concrete.

Two different bacterial strains were utilized by Nosouhain et al (2016) in concrete mix. They examined bacterial effects in harsh sulphate environment and found a 20% increased compressive strength as compared to control along with a subsequent decrease in chloride permeability.

Use of calcium carbonate producing bacteria was done to investigate its influence on light weight aggregate by Balam et al (2017). Investigation concluded decreased water absorption after specimens were subjected to bacterial curing. Calcium carbonate was believed to fill the pores of concrete, precipitated by bacteria. Treated samples also exhibited a 38% increase in compressive strength as compared to control specimens, showed high resistance against chloride penetration and concluded to be less permeable.

Achal et al (2008) reported the improvement in strength and permeation properties of fly ash incorporated mortar and concrete mixes when subjected to treatment with ureolytic bacterial strain Bacillus megaterium.

A 30% increase in compressive strength has been reported by Kalhori et al (2017) as an effect of calcium carbonate precipitating bacteria on mechanical properties of bacterial treated shotcrete specimens. Bacterial presence was also believed to enhance tensile strength and decreased porosity of shotcrete.

Bacterial admixed treated samples showed 29% increased compressive strength as compared to the controlled specimens as reported by Joshi et al (2018) and drastic decrease in permeability properties was also observed.

Thus microbial concrete can be potentially utilized for the enhancement of strength related properties of cementitious materials.

CHAPTER 3
MATERIALS AND METHODS

3.1. Sample Collection

Steel slag is a by-product from iron and steel industries. Samples for the present work were collected from TATA steel industries, Jamshedpur. These samples were passed through pre-determined sieve size of 90 micron and were collected to execute further experimental work.

3.2. Determination of pH of Slag Sample

- 2 g of slag sample was weighed and taken in a clean test tube.
- Added 10 ml of deionised water and put the test tube on incubator shaker for 1hour for proper mixing.
- Then, suspension was allowed to settle down for 5 minutes.
- Reading was recorded when pH meter got stabilized (usually after 30 sec).

3.3. Isolation and Cultivation of Bacterial Species

- For the isolation of urease producing bacteria, 2 gram of steel slag sample was added in 100 ml of autoclaved nutrient broth along with addition of 2% urea (filter sterilized).
- This inoculated culture was incubated at 37°C for 120 hours under shaking condition (130 rpm).
- For enumeration of ureolytic alkaline bacteria, all samples were serially diluted (10^{-1}-10^{-5} dilutions) in saline (NaCl 0.85%) prepared in autoclaved test-tubes.
- 100 µl of each dilution was spreaded by spread plate method on alkaline nutrient agar plates (previously made in 90 mm sterilized petri-plates).
- Inoculated plates were then incubated overnight at 37°C.
- Plates were used for cfu count of colonies and analysed for further experimentation.

3.4. Screening of Ureolytic Bacteria

- Obtained bacterial colonies from the above plates were again plated on fresh nutrient agar plates to obtain pure isolates.

- This was done by Quadrant streaking the selected isolates with the help of inoculation loop.
- Finally random colonies were selected out of all isolated colonies, to be examined for their urea degrading ability, by transferring them onto urea agar plates using urea agar base medium.
- Colonies were transferred by streaking patches of different isolates using inoculation loop and incubated overnight at 37°C.
- The inoculated plates were checked for production of pink colour due to presence of urease on single day incubation and also determined intensity of colour.

3.5. Urease Assay

- All bacterial isolates, showing urease production, were analysed for their urease activity by measuring the breakdown of urea and production of ammonia.
- For this, bacterial isolates were grown in nutrient broth till OD_{600} reaches 0.5 and re-inoculated into fresh nutrient broth and incubated at 37°C under shaking condition (130 rpm).
- After 24 hours, 2 ml of bacterial culture was taken in sterile eppendorfs and pelleted down by centrifuging the culture eppendorfs at 8000 rpm for 5 minutes.
- 250 µl of supernatant was taken in fresh test tubes and added 1 ml of 0.1 M potassium phosphate buffer (pH 8.0) and 2.5 mL of 0.1 M urea solution.
- The obtained reaction mix was vortexed and incubated at 37°C for 5 minutes.
- 1 ml of phenol nitroprusside solution and alkaline hypochlorite solution was then added and incubated for 25 minutes at 37°C.
- The solution was vortexed at the time of addition of solutions.
- Optical density was measured at 626 nm for a continuous of 5 days at interval of 24 hours using UV-Vis spectrophotometer.

3.6. Calcium Carbonate Precipitation Estimation:

- Selected bacterial isolates with highest urease activity were tested for their calcium carbonate precipitation ability.
- 90 ml of nutrient broth was inoculated with 1% of bacterial culture in 100 ml borosil flask.

- Culture was supplemented with 5 ml each of 2% urea (filter-sterilized) and 25mM $CaCl_2$ (filter-sterilized), once the culture got turbid and then the culture was allowed to incubate at 37°C under shaking condition at 130 rpm.
- Inoculated cultures were then observed after 48 hours for calcium carbonate crystals.

3.7. SEM and EDX Analysis of $CaCO_3$ Crystals:

For the preparation of samples for SEM and EDX for morphological analysis, nylon-66 membrane filters of pore size 0.45 µ were placed in filter assembly and autoclaved at 121°C for 15 minutes. The filter membranes containing precipitates were then dried under room temperature and then examined by SEM.

3.8. Extraction of DNA

3.8.1. Isolation of Genomic DNA:

- A single colony of the bacterial isolates was inoculated in 20 ml of nutrient broth in a 250 ml borosil flask.
- The flasks were incubated at 37°C for 16 hours under shaking condition (120 rpm).
- 2.0 ml of the liquid culture was harvested in sterile eppendorfs by centrifuging at 8,000 rpm for 5 min or 12,000 rpm for 1 min.
- The supernatant was decanted while cell pellet was allowed to dry by standing the tubes in inverted position for roughly 1 min.
- An additional step of washing of cell pellet could be done with 200 µl of 10 mM tris-HCl (pH 8.0) at similar centrifuge conditions.
- Then the obtained cell pellet was re-suspended with 0.8 ml of saline-EDTA buffer (0.1 M) thoroughly, added 50 µl of freshly prepared lysozyme (10 mg/ml) and then incubated for 20 min at 37°C.
- Invert mixed the cell suspension several times during duration of incubation.
- Now, 200 µl of 10% SDS was added in cell suspension, mixed well by inverting and incubated in pre-heated water bath at 60°C for 15 min.
- The cell suspension with organic solvents was extracted to remove proteins and cell debris using equal volume of buffer-saturated phenol.
- Upper layer was extracted in separate eppendorfs after centrifuging at 12,000 rpm for 20 min.

- Equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) solution was added to the extracted upper layer. Allowed the tubes to stand still for 10 min and centrifuged, after that, at 13,600 rpm for 25 min.
- Again, extracted the upper aqueous phase gently using pipette and poured to another fresh eppendorfs.
- DNA was allowed to precipitate by adding equal volume of isopropanol and let it stand at -20°C for one hour minimum. Visible white threads confirmed the presence of DNA.
- The tubes were centrifuged at 12,000 rpm for 10 min.
- The supernatant was discarded and suspended the pellet in 500 µl of MQ water.
- Added DNase free RNase (3 µl) to remove any RNA impurities, incubated the resultant solution at 37°C for 30 min with occasional mixing.
- Again, DNA was extracted by adding equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) solution and centrifuged at 12,000 rpm for 25 min.
- The upper aqueous layer was transferred to fresh eppendorfs and precipitated DNA by washing with two volume of 75% ethanol and then with 95% ethanol.
- The DNA was spooled out by completely drying the ethanol from the pellet and re-dissolved the pellet in 30 µl of MQ water.
- The isolated genomic DNA sample can then be stored in refrigerator at 4°C for further use.

3.8.2. Qualitative Analysis of Isolated DNA (Agarose Gel Electrophoresis):

- 100 ng of DNA sample was loaded on 1% agarose gel prepared in 40ml TBE (0.5X) buffer and ethidium bromide was added in the gel.
- After gel casting, DNA sample was loaded in the wells with 6X loading dye.
- Nucleic acids were electrophoresed at 50 volts (3volts/cm) for 100 minutes.
- Gel was visualized under U.V. trans-illuminator.

3.8.3. Quantitative Analysis of Isolated DNA:

Quantitative analysis was done by using a Nano drop 1000 spectrophotometer (Thermo scientific, Wilmington, DE). Quantity of DNA sample was evaluated by measurement of A260/280 ratio. Ideally, A260/A280 ratio should be in range of 1.8-2.0. Ratio less than 1.8 indicates protein or phenol contamination, while ratio greater than 2.0 indicates the presence of RNA.

3.8.4. Amplification of 16S rDNA samples:

For a gene of 1.5 kb, primers used were
Forward primer: 5'-AGA GTT TGA TCC TGG CTC AG-3'
Reverse primer 5'- ACG GGC GGT GTG TTC-3'

COMPOSITIONS OF REACTION MIX:

Table 3.1: Composition of reaction mixture of 16S rDNA amplification

Components	**Concentration**	**Volume for 1 reaction (in (µl)**
MQ Water	-	12.2
PCR Buffer	10 X	2
dNTPs		1.5
DNA template	10 ng/µl	1
$MgCl_2$	1.5 mM	1
Forward primer	0.2 mM	1
Reverse primer	0.2 mM	1
Taq polymerase	2.5 U/µl	0.3

REACTION CONDITIONS:

Table 3.2: Reaction conditions for 16S rDNA amplification

Cycle	**Temperature**	**Duration**
Initial Denaturation	92^{o}C	2 minute
Denaturation	92^{o}C	1 minute
Annealing	50^{o}C	30 second
Elongation	72^{o}C	2 minute
Final Elongation	72^{o}C	7 minute

PROTOCOL:

- Amplification of the above 20 µl of reaction mixture was performed in GenAmp 2700 thermocycler (Applied bio-system USA) with 35 cycles of amplification.
- A control reaction containing no DNA template was included in each amplification process so as to check for the presence of contamination of reagents and buffer.
- Aliquots (6µl) of amplification products were electrophoresed in 1% agarose gel and visualized on a UV transilluminator.

3.8.5. PCR products purification:

- Purification was done using QIAquick gel extraction kit (Qiagen Inc., USA).
- PCR products were purified by agarose gel (0.8%) electrophoresis prior to cloning. Besides removing surplus primers, nucleotides and salts, this method possessed the advantage that incomplete (shorter) amplification fragment was removed prior to the cloning.
- Protocol was as per manufacturer's instructions.
- Purified PCR products were then suspended in 30 µl MQ water and stored till further use.

3.8.6. RFLP of the Purified PCR Products:

- Restriction Fragment Length Polymorphism (RFLP) is a technique which is carried out to exploit polymorphism between two homologous DNA sequences. This includes visual interpretation of DNA bands of the restriction digested samples.

Table 3.3: Components of reaction mixture of RFLP technique

Components	Volume in µl
PCR product	5 (0.5-1 µg of purified DNA)
Nuclease-free H_2O	12
Buffer	2
Restriction enzyme (U/µl)	1

- The reaction mixture was spun gently using mini-centrifuge and incubated up to 16 hours at different temperatures respective to each restriction enzyme (Thermo-Fisher scientific) is depicted in table (3.4).

- After carrying out with the above procedure of reaction mixture, the obtained samples were then analysed on 1.2 % agarose gel.
- Electrophoresis was performed at (3 volts / cm) and run for 3 hours.
- Compositions of buffers for each restriction enzyme are mentioned in appendix I.

Table 3.4: Restriction enzymes along with their restriction sites and other characteristics

Restriction enzyme	Restriction site	Compatible Buffer	Activation temperature & time	Inactivation temperature & time
Hpa II	C*C G G	Tango buffer	37°C (1-16 hr)	65°C (20 min)
Alu I	A G*C T	Tango buffer	37°C (1-16 hr)	65°C (20 min)
Taq I	T*C G A	1 X buffer Taq I	65°C (1-16 hr)	0.8 µl 0.5 M EDTA and mix

3.8.7. Ligation of 16S rRNA in pMD20T vector:

The purified PCR products were cloned using Takara cloning kit as per manufacturer's instructions. The obtained ligated product was then stored at -20°C until further use transformation. The reaction was made as follows:

Table 3.4: Ligation reaction mixture for TA cloning

Components	volume (in µl)
Plasmid (PMD20 T)	1
Insert	2
Mighty mix	5
MQ water	2

3.8.8. Genetic Transformation of Bacteria:

(All genetic operations were carried out as per standard protocol by Sambrook)

- 100 µl of *E. coli* DH10 β cells were inoculated in 20 ml Luria broth in 250 ml borosil flasks and incubated it for 16 hours at 37^{o}C under shaking condition (120 rpm).
- Aseptically transferred 1% of the above saturated culture into a fresh 20 ml Luria broth and incubated at 37^{o}C for 2 hours with vigorous shaking at 120 rpm.
- O.D. was taken at 600 nm which should be 0.5 so as to confirm growth of bacteria.

- The culture was transferred to sterile ice-cold falcon (50 ml).
- The falcons were placed on ice for 10 more minutes.
- The cells were recovered by centrifugation at 5000 rpm for 10 minutes at 4^oC.
- The media i.e., supernatant was decanted and allowed it to stand for 1 minute.
- Now, the cells were re-suspended in 1 ml of ice cold 0.1 M $CaCl_2$ and washed 2-3 times by centrifuge conditions of 4000 rpm for 10 minutes at 4^oC.
- The cells were then stored on ice for 12 hours minimum.
- Aliquots of 100 µl suspension of competent cells were transferred to sterile and pre-chilled eppendorfs and added 80% glycerol to the cells.

3.8.9. Blue-White Screening of Competent Cells:

- Previously cloned product was transferred in 100µl of E. coli competent cells including a control containing no plasmid and another control with insert.
- The contents of the eppendorfs were mixed gently.
- The eppendorfs were stored on ice for 30 minutes for the binding of the plasmids.
- The eppendorfs were then incubated in water bath pre-heated at 42^oC for 2 minutes.
- Eppendorfs were transferred on ice rapidly for 1-2 minutes for heat shock.
- Then, 1 ml of Luria broth was added and incubated at 37^oC for 45-60 minutes to allow bacteria to recover and express antibiotic resistance marker encoded by plasmid.
- 100 µl of transformed cells were spreaded on LA + ampicillin (100µg/ml) plates by spread plate method.
- The plates were pre-spreaded with 40 µl of each isopropyl beta-D-thiogalactopyranoside (IPTG) and X-gal (5-bromo-4-chloro-3-indoyl β-D galactosidase).
- X-gal and IPTG were used for the screening of colonies containing a recombinant plasmid.
- The plates spreaded with transformed cells were then incubated overnight at 37^oC in upright position.
- The plates were then checked for the appearance of either recombinant or non-recombinant colonies after 16-20 hours.
- Random white colonies were picked i.e., 3 or 4 for each isolate and the colony PCR was performed by using M_{13} primers.

- Positive clones were further grown in LB+ ampicillin media and were subjected to plasmid DNA isolation.

M_{13} primers used were:

- Forward primer: 5'-GGT TTT CCC AGT CAC GAC-3'
- Reverse primer 5'- GGA AAC AGC TAT GAC CATG-3'

COMPOSITION OF REACTION MIXTURE:

The reaction mixture was prepared and heated at 98^{o}C to allow the lysis of cells and also reaction mix without DNA template can be used as negative control in analysis of DNA bands.

Table 3.5: Components and volume of reaction mixture for colony PCR

COMPONENTS	**VOLUME FOR 1 REACTION (in µl)**
MQ water	6
Buffer	2
dNTPs	1.5
$MgCl_2$	1
Forward Primer	1
Reverse Primer	1
Template	3
Taq polymerase	0.3

REACTION CONDITIONS FOR PCR:

Table 3.6: Reaction conditions for colony PCR

Cycle	**Temperature**	**Duration**
Initial Denaturation	92^{o}C	2 minute
Denaturation	92^{o}C	1 minute
Annealing	50^{o}C	30 second
Elongation	72^{o}C	2 minute
Final Elongation	72^{o}C	7 minute

3.8.10. Plasmid DNA Isolation (alkali lysis method):

- 10 ml of Luria Broth was prepared in a test tube and sterilized using autoclave.
- Ampicillin (100µg/l) was added to the medium after its temperature reaches up to 40-50°C.
- A single bacterial colony was inoculated in the luria broth in test tube and incubated at 37^{o}C for 16-24 hours.
- 5 ml of culture was taken and centrifuged at 8000 rpm for 5 minutes to pellet down the cell culture.
- 200 µl of ice-cold solution I (appendix I) was added and mixed using vortex (to ensure that bacterial pellet was completely dispersed in the solution).
- It was allowed to stand at room temperature for 3 minutes.
- 400 µl of freshly prepared solution II (appendix I) was added and mixed by inversion.
- The tubes were kept on ice for 10 minutes.
- Then, 300 µl of ice-cold solution III (appendix I) was added and invert mixed gently.
- The tubes were placed on ice for 10 more minutes.
- The contents were then centrifuged at 12000 rpm for 10 minutes at 4^{o}C.
- The supernatant was transferred to a fresh tube and added equal volume of phenol: chloroform: isoamyl alcohol (25:24:1).
- The suspension was then again centrifuged at 10,000 rpm for 20 minutes.
- Upper aqueous layer was then transferred to a fresh tube and equal volume of isopropanol was added to it.
- Gently inverted the tubes few times and allowed them to stand for 10 minutes at room temperature.
- The contents were again centrifuged at 8000 rpm for 10 minutes and discarded the supernatant.
- 300 µl of 70% ethanol was added to the cell pellet for washing and centrifuged at 8000 rpm for 5 minutes.
- The supernatant was decanted and the pellet was allowed to air-dry.
- The obtained pellet was finally suspended in 30 µl of MQ water.
- After plasmid isolation, again colony PCR was performed and the samples were checked on agarose gel (1.0% w/v) electrophoresis.

3.8.11. 16s rDNA Sequencing:

The 16S rDNA inserts were sequenced for both strands using M_{13} forward and reverse primers, used for pMD20T vector. The sequences were generated by using an applied bio systems automatic sequencer (DNA sequencing facility, Department of Biochemistry, South Campus, Delhi University, New Delhi, India).

3.8.12. Analysis of Sequenced Data:

The 16S rDNA gene sequences of isolates were compared with those available in EZ taxonomy databases using blastN program. The strains with closely related sequences were aligned by using multalin program. Phylogenetic tree was constructed by maximum parsimony method by using MEGA-7 program (Tamura et al, 2011)

3.9. Physiological Characterization

3.9.1. Alkalinity Test:

Different pH conditions (pH 7.0, 9.0, 10.0, 11.0) were maintained in nutrient broth to monitor the growth rate of bacterial isolates. Growth under different pH conditions was recorded by measuring the absorbance after every one hour of inoculation at 600. Cultures were grown at steady temperature i.e., 37^{o}C.

3.10. Morphological and Biochemical Characterization

3.10.1. Gram Staining:

A thin bacterial smear was heat fixed on clean slide. Flooded each smear with crystal violet for 30 seconds and then washed using distilled water. Added Gram's iodine solution for 60 seconds and Ethyl alcohol was added drop wise. Rinsed it with distilled water and added secondary stain, safranin for 1 minute and then again rinsed it with water. The slides were air-dried and observed under microscope by using oil- immersion objective. If the bacteria appeared purple in colour (retains primary stain), then they were considered gram-positive bacteria while the bacteria which appeared reddish-pink colour (retains secondary stain) were considered as gram-negative bacteria.

3.10.2. Motility test:

A semi-solid media of nutrient broth with 0.4% agar was made and autoclaved. The media was poured in the test tubes and bacterial colonies were inoculated in the media with the help of an inoculating loop. The tubes were incubated at 37^{o}C for 24 hours and the depth of turbidity was then analysed.

3.10.3. Catalase test:

A small bacterial colony was placed on the clean slide with the help of an inoculation loop. Few drops of hydrogen peroxide (H_2O_2) were added and appearance of bubbles indicated the evolution of Oxygen i.e., Positive results while no bubbling or a few scattered bubbles inferred negative results. Later appearance of bubbles was ignored.

3.10.4. Oxidase test:

This test was performed by using oxidase discs manufactured by HiMedia, India. A pure isolated colony was taken on clean glass slide and oxidase test discs were placed on to the slide above bacterial colony. Reaction was observed within 5-10 seconds at 25-30°C. A positive reaction showed deep purplish blue colouration. A change after than 10 seconds or no change at all was considered as negative reaction.

3.10.5. Nitrate reduction test:

Nitrate agar is used to test the ability of organisms to reduce nitrate to nitrite using enzyme nitrate reductase. For this test, nitrate discs manufactured by HiMedia, India were used. The bacterial isolates were grown on nutrient agar plates. Placed Part A (disc) on bacterial colony and added a drop or two of part B (rehydrating fluid) on the disc. The nitrate reduction was observed after incubation at 37°C for 18-24 hours. Red or pink colour formation on addition of discs indicated positive reaction while negative reaction indicated no colour change.

3.10.6. Fermentation of Carbon substrate by bacterial isolates:

The fermentation broth contained the same ingredients as that of nutrient broth. Additionally, a specific carbohydrate and a pH indicator (phenol red) were also added. Phenol red at or below pH 6.8 turns out from red to yellow because of the production of organic acids.

(Carbohydrates used were glucose, lactose, maltose and mannitol).

Fermentation broth was taken in test tubes and durham tubes were added in an inverted position. The media was sterilized by autoclaving at 121^{o}C for 3 minutes. The media was inoculated with the test organism and kept one tube uninoculated as a comparative control and the tubes (including control) were incubated at 37^{o}C for 24 – 48 hours. The changes in inoculated tubes were compared with uninoculated one (control). Change in colour of inoculated tubes indicated the production of acid alone. But appearance of bubbles along with colour change confirmed the production of acid along with gas i.e., fermentation.

3.10.7. Hiaureus coagulation confirmation test (HiMedia, India):

- The test is mainly performed for rapid detection of coagulase positive pathogenic *Staphylococcus aureus.*
- The test was performed as per manufacturer's instructions.
- Clot formation in the inoculated tube was considered as positive reaction i.e., presence of *Staphylococcus aureus*.

3.10.8. Mannitol Salt Agar Test:

This test is done for selection and sub-culturing of pathogenic Staphylococci. Plates of mannitol salt agar were prepared and bacterial isolates to be tested were inoculated using quadrant streaking method. Inoculated plates were then incubated at 37^{o}C for 1-2 days. Appearance of yellow-white colonies indicated positive reaction while no colour change indicated negative reaction.

3.11. Evaluation of bacterial isolates for enhancement of strength of cementitious materials

3.11.1. Preparation of cement paste:

This experimental program was conducted to analyse the influence of addition of slag powder on the initial and final setting time of cement mixtures. The variation in initial and final setting time of cement slag paste was investigated by using Vicat apparatus as per IS 4031: 1988. Briefly, water required to produce cement paste of standard consistency, was first

determined by IS 4031: 1988. Then paste was prepared by using 0.85 times the water required to provide standard consistency to the paste. Accordingly, 27.62% water was added and standard consistency of the cement paste was determined to be 32.5%.

Mainly, 5 kinds of cement pastes were prepared. Composition of various cement mixes prepared is shown in table (3.7). Control paste included the mixture of only cement and water. 20 Cement Slag (CS) paste was prepared by mixing 320 g of cement with 80 g of slag powder in it. 30 CS consisted of 120 g of slag powder and subsequently cement composition was decreased to 280 g. 40 CS and 50 CS included 160 and 200 g of slag respectively. The consistency of all the cement mixes was kept same.

Table 3.7: Compositions of cement and slag in cement slag paste mixtures.

Cement mixes	Cement (g)	Slag (g)	Water (g)
Control	400	-	110.5
20CS	320	80	110.5
30CS	280	120	110.5
40CS	240	160	110.5
50CS	200	200	110.5

3.11.2. Preparation of mortar specimens:

Mortar specimens were prepared by using cement: slag: sand with water to cement ratio of 0.5. The slag powder was added by replacing the amount of cement at 20%, 30%, 40% and 50% by weight. Mortar specimens with partial replacement of cement with 20%, 30%, 40% and 50% slag powder are represented in table (3.8). Cement, sand and slag powder were mixed thoroughly for 2 min before adding water. The fresh mix prepared in plastic stage was immediately transferred to iron moulds (50 mm × 50 mm × 50 mm). After the casting was done, specimens were allowed to remain in iron moulds (figure 3.1) and kept at room temperature for 24 hours. Afterwards, specimens were de-moulded and cured till the testing period of 28 days. 2 different curing conditions as mentioned in table (3.9) were performed as pictorially represented in figure (3.2).

Table 3.8: Compositions of various components used in preparation of mortar specimens

Ingredients		Control	Cement slag specimens (CS)				Cement slag bacterial treated specimens (CSBT)			
			20CS	30CS	40CS	50CS	20CSBT	30CSBT	40CSBT	50CSBT
CEMENT		74.6	59.73	52.26	44.8	37.33	59.73	52.26	44.8	37.33
SLAG	20	-	14.93	-	-	-	14.93	-	-	-
	30	-	-	22.4	-	-	-	22.4	-	-
	40	-	-	-	29.86	-	-	-	29.86	-
	50	-	-	-	-	37.33	-	-	-	37.33
SAND		223.6	223.6	223.6	223.6	223.6	223.6	223.6	223.6	223.6
WATER		37.3	37.3	37.3	37.3	37.3	37.3	37.3	37.3	37.3

Figure 3.1: Mortar samples prepared for different testing procedures

Table 3.9: Methods for curing of control specimens, cement slag (CS) specimens and cement slag bacterial treated (CSBT) specimens

Specimen	Material used	Method of curing
Control	cement: sand w/c = 0.5	Water curing for 28 days
Cement slag specimen (CS)	cement: slag: sand w/c = 0.5	Submersion in water for 28 days
Cement slag bacterial treated specimen (CSBT)	cement: slag: sand w/c = 0.5	Submerged in nutrient broth, urea, $CaCl_2$ and bacterial culture for 28days

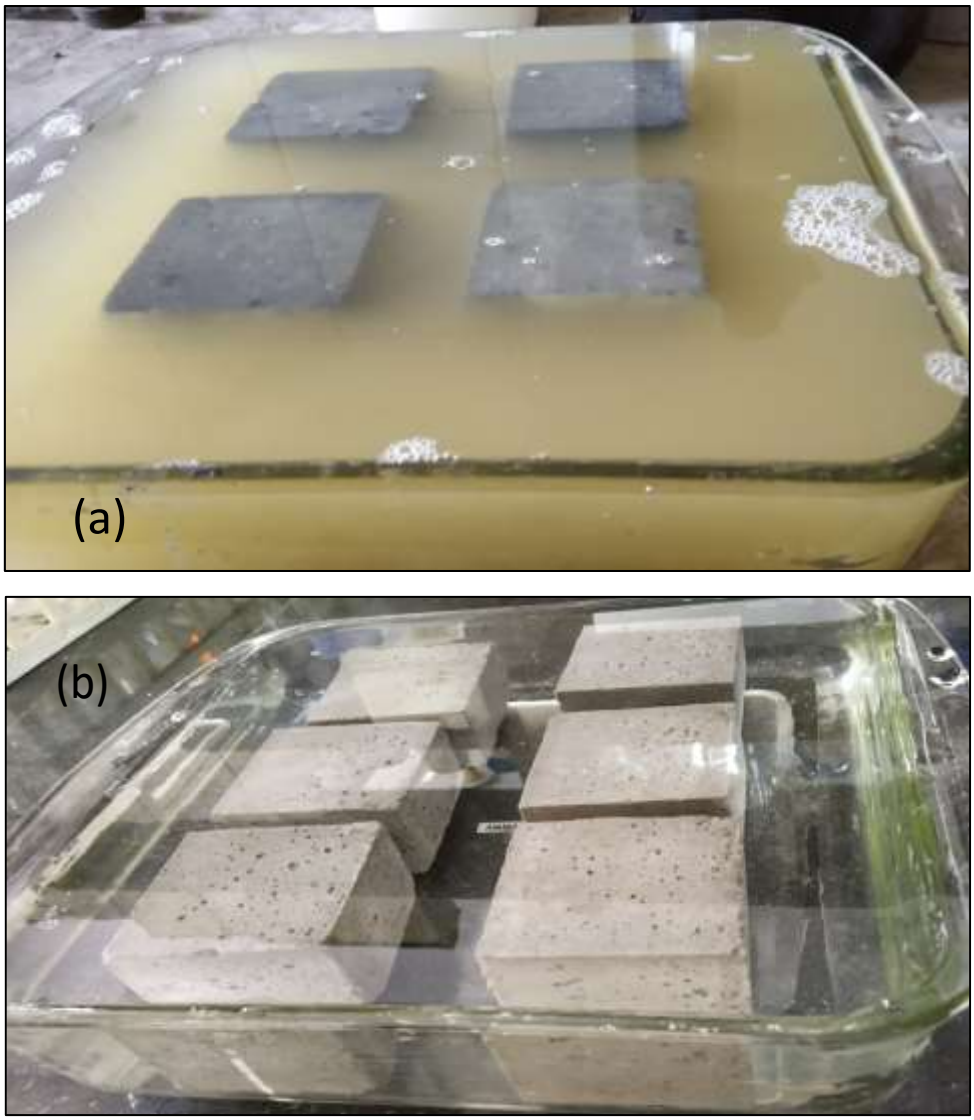

Figure 3.2: Curing method (a) cement slag bacterial treated (CSBT) specimens and (b) Cement slag (CS) specimens

3.11.3 Testing procedures

3.11.3.1. Evaluation of Compressive Strength of Mortar Mixes

Mortar specimens of 50x50x50 mm dimension were casted to investigate the compressive strength. The effect of incorporation of slag powder at different ratios on mechanical properties of mortar cubes was studied at the age of 28 days of curing as per BIS 516: 1959. It was done using auto compression testing machine COMPTEST 3000 (figure 3.3). Mainly three different kinds of specimens were prepared as control, cement slag (CS) specimens and cement slag bacterial treated (CSBT) specimens. The average of three specimens was taken as the compressive strength of the mix.

Scanning Electron Microscopy (SEM) (ZEISS EVO 50) analysis was done on mortar specimens at the age of 28 days. The elemental composition of micro-structural analysis was identified with Energy Dispersive X-ray spectroscopy (EDX). For conducting SEM and EDX analysis, small pieces of samples from different depths were collected. Samples were finally polished and gold coated with sputter coating. To disperse excess charge from the sample, a thin coating of carbon was applied on the polished surface.

Figure 3.3: Autocompression testing machine COMPTEST 3000.

3.11.3.2. Water Absorption Test:

A sorptivity test is a test to study resistance of mortar specimens towards penetration of water inside the specimens. It is based on the RILEM 25 PEM (II-6) and was followed for all mortar cubes. Similar protocol was carried out to prepare samples for this test as done in case of compressive strength. The mortar specimens were submerged completely in 10±1 mm water and were cured for 28 days.

Before checking for the sorptivity, samples were oven dried at 45°C for 24 hours and were then coated with epoxy on 5 out of 6 faces of cubes. Initial weight of samples was recorded. Uncoated face of the cube was then exposed to water and mass increase of specimen due to water absorption was recorded at different time intervals i.e., 15 min, 30 min, 1 hour, 1.5hour, 3hour, 5hour, 24hour and 48 hours. The specimens were immersed (figure 3.4) immediately after measurement. The sorptivity coefficient was obtained by plotting graph between Q/A against square root of time.

The expression used for calculating the same is

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

Here, Q is amount of water absorbed [cm^3]

A is the cross-sectional area of sample immersed in water.

t is the time in seconds and,

k was calculated from the linear slope.

Slope of graph was then taken as the sorptivity of the sample.

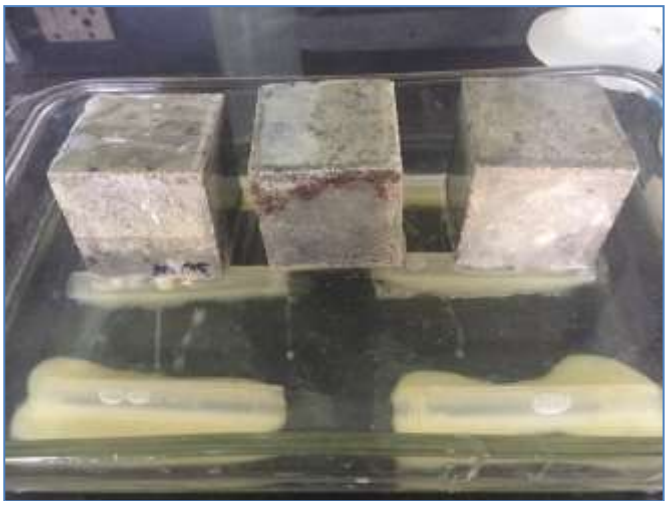

Figure 3.4: Water absorption test of epoxy coated mortar specimens.

3.11.4. Statistical analysis:

All the experiments of mortar testing performed above were performed in hexaplicates and mean values were considered as final results. The data were analysed by using Graph Pad Prism (5.03) software.

CHAPTER 4
RESULTS

4.1. Steel slag sample

Sample of steel slag collected from TATA steels, Jamshedpur and used for research in present study is depicted in figure (4.1).

Figure 4.1: Sample of steel slag.

4.2. pH of steel slag sample

pH of the sample was recorded to determine the alkalinity of the sample so as to optimize the parameters to be used with the sample. The pH recorded from the pH meter after making a suspension came out to be 12.26 ± 0.2.

4.3. Isolation and cultivation of bacteria

Nutrient broth was supplemented with 2 g of steel slag sample and 2% filter-sterilised urea to isolate alkaline bacteria from it. The sample inoculated culture flask was incubated for 120 hours. Culture flask showed turbidity after 4 hours of inoculation

After incubation, serial dilution was carried out and numerous numbers of colonies were observed after spreading each dilution on nutrient agar plates. The number of colonies

decreased on increasing the factor of dilution. Colonies observed after spread plate method are presented in figure (4.2)

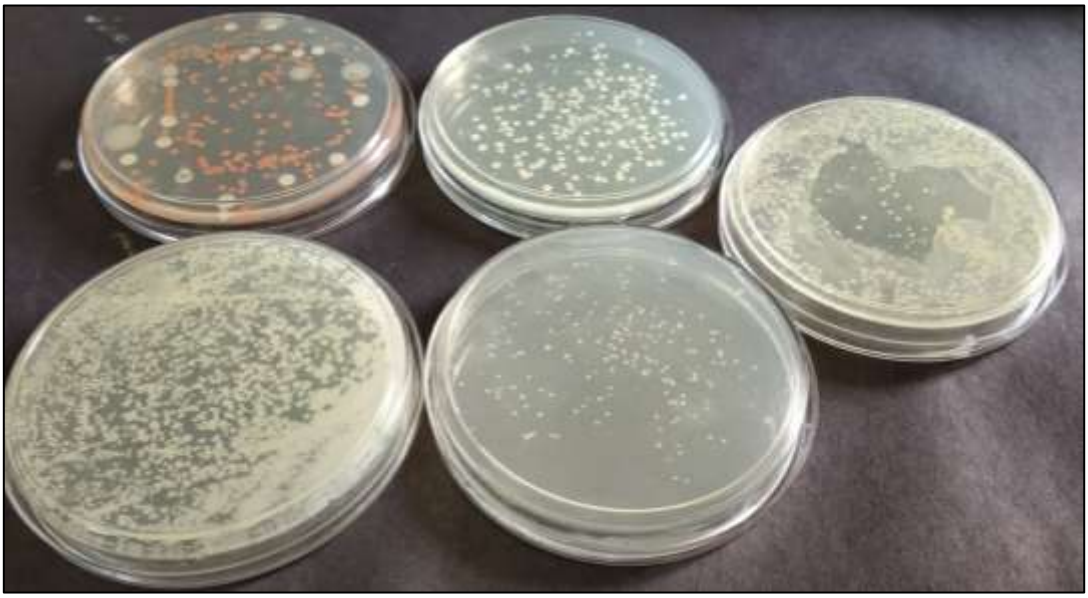

Figure 4.2: Numerous isolated colonies from inoculated steel slag sample

The colonies which were observed varies in morphological characteristics like shape, size, colour and number of colonies per plate. Appearance of different kinds of colonies demonstrated the possibility of presence of numerous species of bacteria in steel slag sample.

4.4. Screening of ureolytic bacteria

Out of all the colonies isolated, inoculation of random 20 colonies (UAB 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20) on urea agar base plates was done. Here, UAB stands for ureolytic alkaline bacteria. The urea and phenol red (pH indicator) in urea agar base medium composition were key indicators for confirmation of ureolytic activity of bacterial species. As the bacterial strain utilized urea and produced ammonia due to enzymatic reaction in presence of urease enzyme, it caused alkaline pH of media and phenol red indicated the pH change by turning the orange-yellow urea agar plate into pink coloured plate. This visible indication of change of colour of media confirmed the ureolytic activity of bacteria. The colonies which do not showed pink colour were considered to be non-ureolytic. Random colonies inoculated on urea agar base plates were tested for formation of pink colour as shown in Figure (4.3). Pink colour was observed within a day of incubation and observations were recorded.

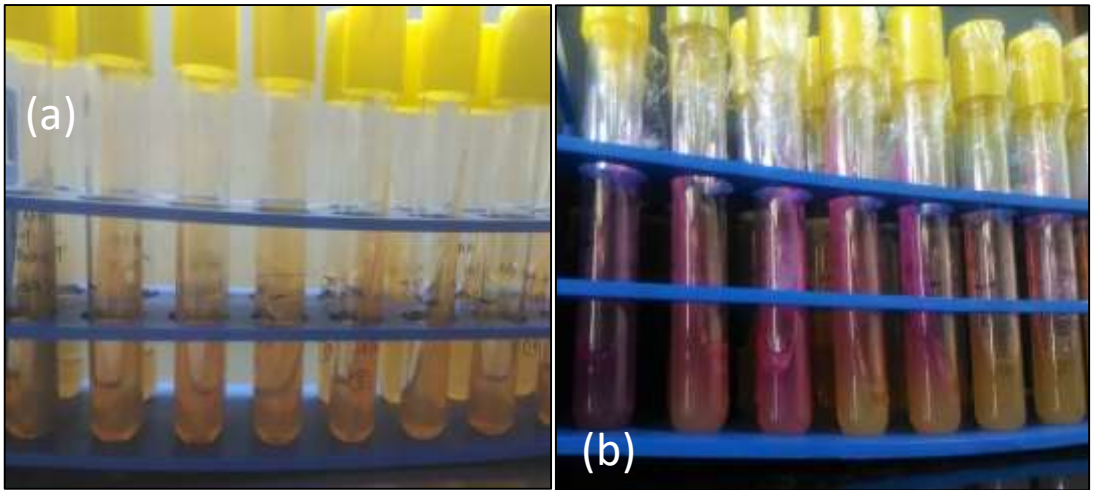

Figure 4.3: Urea agar base slants (a) orange-yellow coloured slants before inoculation, (b) pink colour formation after inoculation of bacterial colonies and incubation

After testing of 20 bacterial colonies out of large no. of colonies obtained on nutrient agar plates, 12 isolates (UAB1, UAB2, UAB3, UAB4, UAB5, UAB6, UAB7, UAB9, UAB16, UAB17, UAB18, UAB19) showed ureolytic activity in slants and were re-inoculated on urea agar plates, as shown in figure (4.4) for confirmation of their ureolytic activity.

Figure 4.4: Pink colour production by selected bacterial strains

4.5. Urease assay

Out of 20 bacterial strains, 12 bacterial colonies (UAB 1, 2, 3, 4, 5, 6, 7, 9, 16, 17, 18, 19) confirmed their ureolytic activity. On the basis of early production of pink colour, these 12 bacterial isolates were selected for the detection of presence of urease enzyme. It was basically a colorimetric assay in which alkaline hypochlorite detects the production of ammonium carbonate and indication was done by production of blue colour as visible in

figure (4.5). Intensity of colour produced was proportional to the concentration of urease present and was analysed by spectrophotometric analysis.

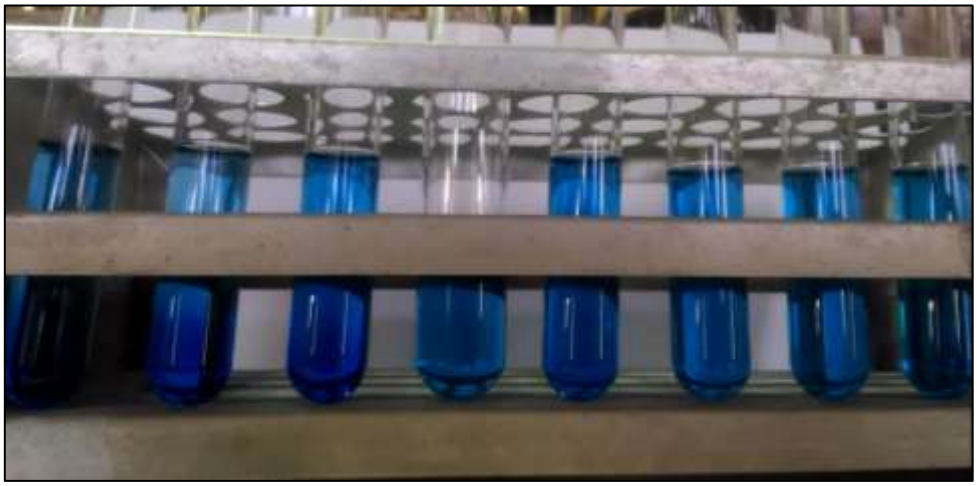

Figure 4.5: Blue colour indication due to production of ammonia in urease assay

On the basis of intensity of blue colour and concentration of urease (in U/ml) of the strains after 5 days, strains with maximum urease activity (figure 4.6) were selected. Best ureolytic strains with maximum urease activity were believed to show high amount of calcium carbonate precipitation.

Table 4.1: Concentrations of urease activity (in U/ml) of different bacterial isolates

Bacterial Isolates / Time (in hours)	**24**	**48**	**72**	**96**
UAB 1	301.28±0.5	479.35±0.4	677.40±0.5	532.42±0.5
UAB 2	336.95±0.3	647.72±0.5	885.216±0.4	702.14±0.5
UAB 3	385.98±0.5	405.0±0.2	825.84±0.5	806.05±0.4
UAB 4	307.31±0.2	392.4±0.5	1033.6±0.2	791.2±0.1
UAB 5	451.25±0.4	543.8±0.2	672.46±0.1	1053.4±03
UAB 6	64.35±0.8	716.99±0.7	736.7±0.5	880.2±0.7
UAB 7	302.8±0.6	499.2±0.5	538.80±0.2	573.5±0.2
UAB 9	375.09±0.4	573.5±0.4	791.2±0.3	716.95±0.5
UAB 16	1013.85±0.7	994.06±0.5	1003.9±0.4	1429.9±0.4
UAB 17	479.45±0.5	528.9±0.6	687.3±0.4	1112.8±0.3
UAB 18	736.75±0.1	820.8±0.1	716.9±0.2	1053.4±0.1
UAB 19	1028.7±0.5	647.72±0.3	558.66±0.4	1394.84±0.5

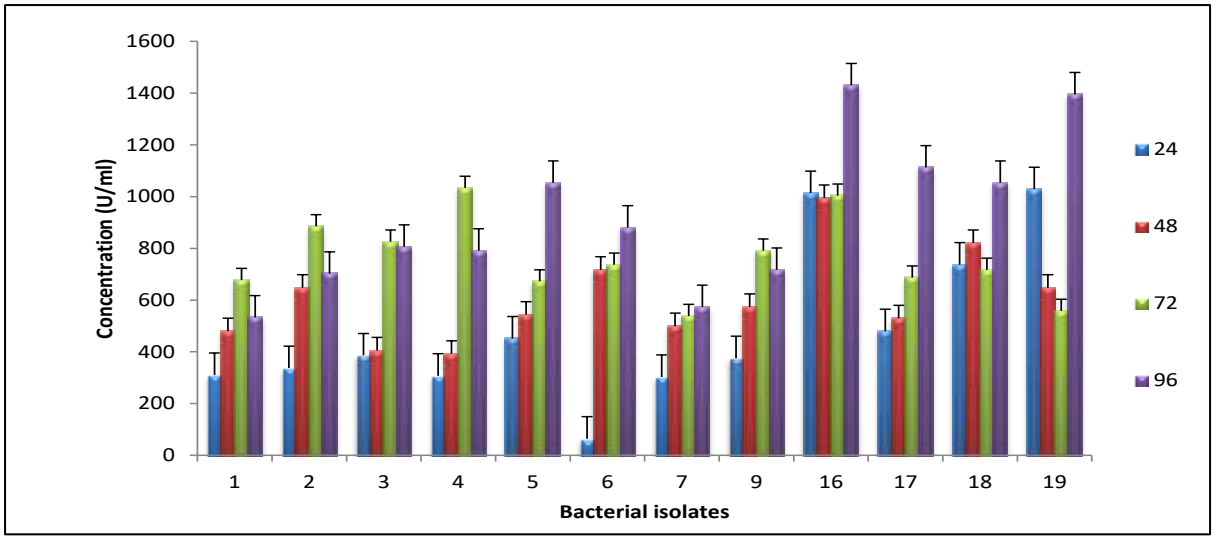

Figure 4.6: Urease activity of various bacterial isolates represented in terms of concentration in U/ml.

4.6. Precipitates of calcium carbonate

As per the optical densities of urease assay of 12 bacterial strains, 4 bacterial isolates with maximum urease activity were finally selected. In the presence of urea and calcium chloride, bacterial strains were believed to precipitate calcium carbonate.

The inoculated bacterial strains in nutrient broth supplemented with urea and calcium chloride showed calcium carbonate precipitation at the walls of the flasks depicted in figure (4.7). Filtrate of this culture over nylon filters (pore size 0.25 µ) represented in figure (4.8) were used for SEM analysis.

Figure 4.7: Calcium carbonate precipitated on the walls of culture flask.

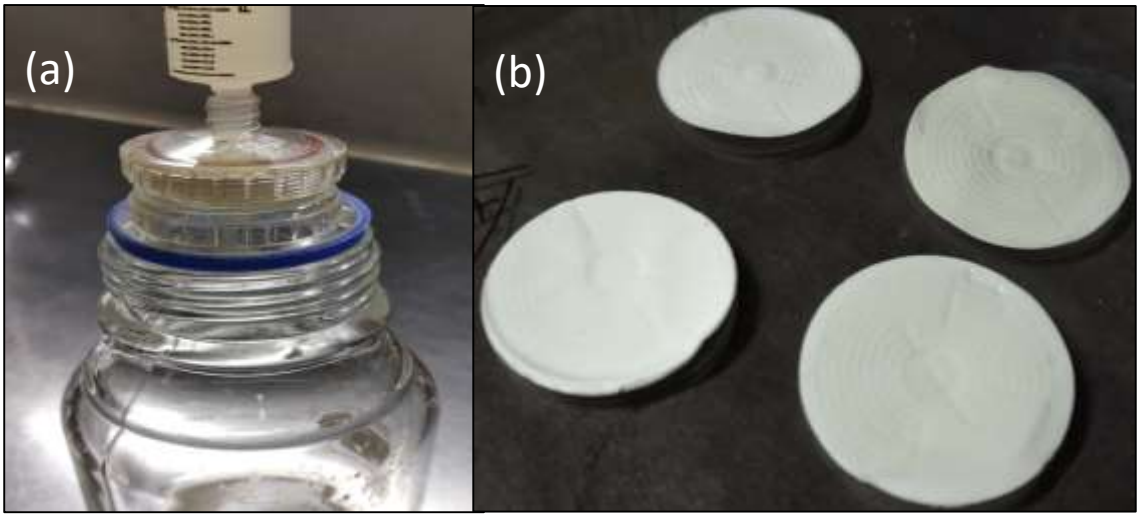

Figure 4.8: (a) Filter assembly for precipitated bacterial culture, (b) precipitates obtained on filter paper.

Precipitates of calcium carbonate were also be seen by streaking the bacterial strain on nutrient agar plate previously containing urea and $CaCl_2$ in required amount. The streaked plates with bacterial isolates after incubation showed yellow coloured colonies. These colonies showed distinct morphology as compared to normal nutrient agar plate colonies which are also shown in figure (4.9).

Figure 4.9: Calcium carbonate precipitation (a) yellow colonies of bacterial strain on NA+ urea+ $CaCl_2$ plates, (b) white colonies of bacterial strain on NA plate (control)

4.7. SEM-EDX analysis of calcite precipitates

Scanning electron microscope was used to determine the crystal structure and morphology of calcium carbonate precipitated by selected bacterial strains. EDX was done to determine the content of calcium carbonate present and to analyse the crystal composition.

SEM images obtained for the 4 selected bacterial strains are presented in figure (4.10).

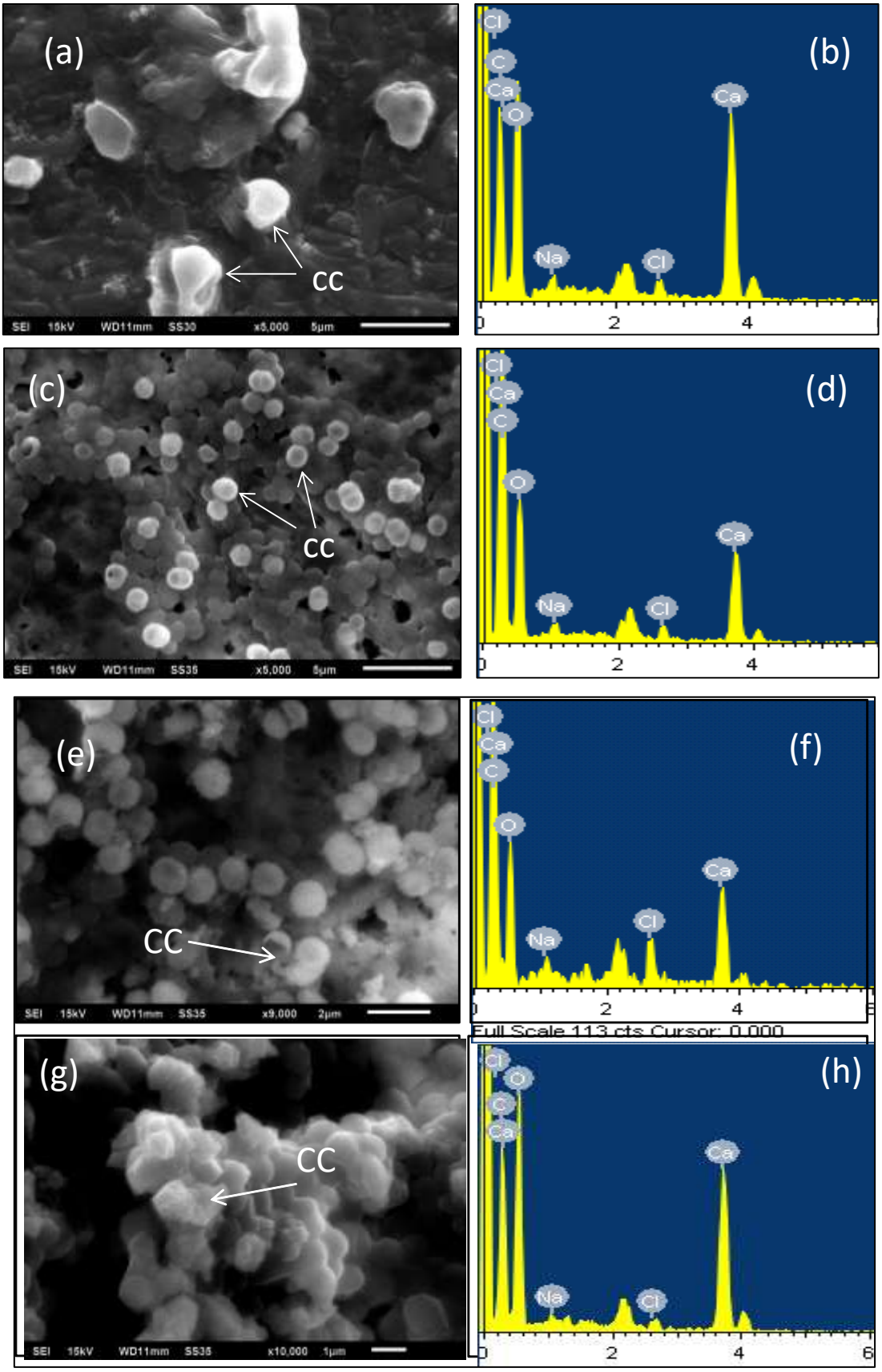

Figure 4.10: SEM-EDX images represent $CaCO_3$ crystals in bacterial nutrient broth sample (a),(b) UAB 5 (c),(d) UAB 9 (e),(f) UAB 16 (g),(h) UAB 18

4.8. Molecular Analysis of Ureolytic Bacteria

4.8.1. Genomic DNA analysis

After screening of 4 strains (UAB5, UAB9, UAB16 and UAB18) for their calcite crystal precipitation, all were subjected to genomic DNA isolation. Extracted genomic was visualised by agarose gel electrophoresis and bands of DNA in gel (figure 4.11) were compared with Molecular weight marker (1.5 kb) to detect the size of DNA of all the bacterial strains. An agarose gel of 0.8% was chosen because smaller pore size of the gel would hinder the movement of DNA across the gel. DNA bands observed by this quantitative method shows purity of DNA extracted. Smearing was possibly observed due to some content of RNA present in the bands.

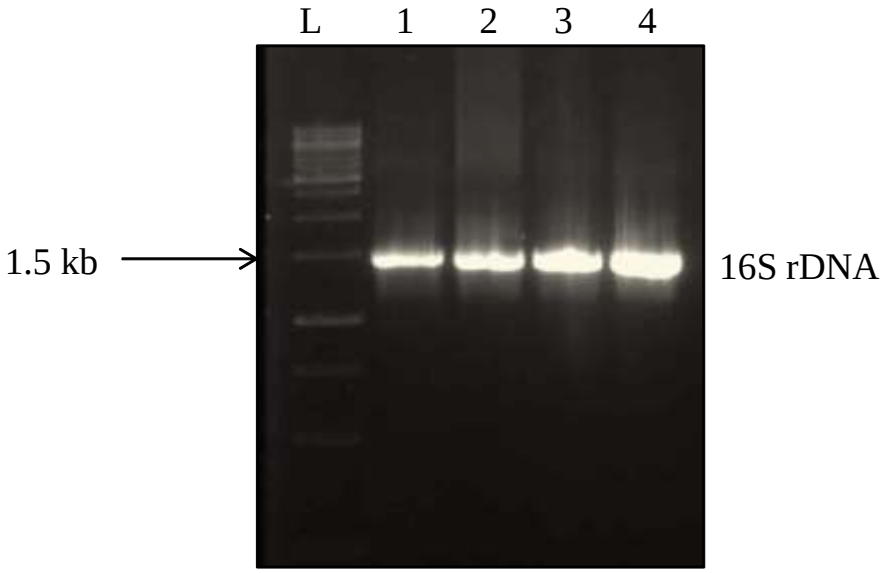

Figure 4.11: Isolated 16S rDNA of bacterial isolates. Lane 1-4: UAB5, UAB 9, UAB 16, UAB 18 and Lane L: 1 kb ladder (Fermentas)

4.8.2. Quantitative analysis of isolated DNA

To quantify and check for DNA purity, Nano-drop spectrophotometer was used. A260/280 of double stranded DNA sample is believed to be in range of 1.8 - 2.0 as less than 1.8 indicates protein contamination, while more than 2.0 indicate the presence of RNA. The table (4.2) shows the ng/µl concentration and A260/280 ratio of all the DNA samples. The A260/280 ratio reflected that DNA was present in permissible range of purity and least concentration of contaminations are assumed to be present which were then removed by purification process.

Table 4.2: Nanodrop spectrometer analysed DNA concentrations and absorbance ratio

DNA samples	Concentration of DNA (ng/ µl)	A 260/280 ratio
UAB 5	117.9	1.89
UAB 9	177.6	1.83
UAB 16	168.2	1.84
UAB 18	213.2	1.86

4.8.3. Amplification of 16S rDNA strands and purification

As a large amount of nucleic acid was required to study a specific region of DNA, amplification was performed to increase the concentration of DNA present in 1 µl. 4 reactions for each DNA sample were amplified and then all the amplified products were pooled to get increased concentration of amplified products. A small amount of this amplified product was then analysed with the help of gel electrophoresis to confirm efficient amplification. The gel bands in figure (4.12) confirmed that all the bacterial isolates had similar amplicon sizes.

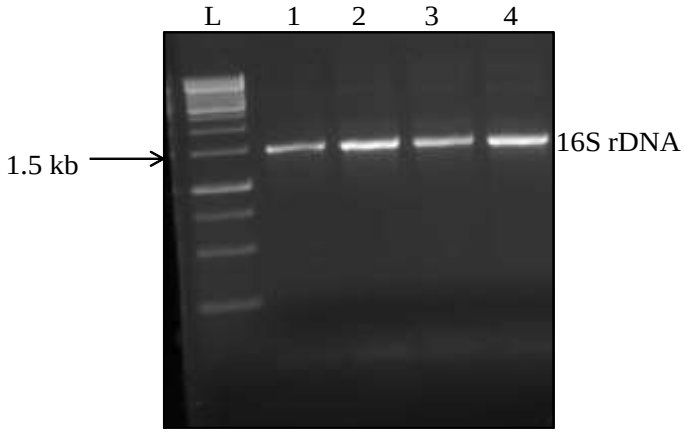

Figure 4.12: 16S rDNA amplified products of bacterial isolates. Lane 1-4: UAB 5, UAB 9, UAB 16, UAB 18 and Lane L: 1 kb ladder (Fermentas)

Purification of these amplified products before cloning was done so as to minimize contamination of proteins or RNA. The QIAquick PCR purification kit (Qiagen) enabled rapid and efficient purification of all amplified products. This purification ensured removal of all kinds of primers, unincorporated labelled nucleotides and enzymes etc. from the reaction mixture. The purified DNA was used for ligation and cloning processes.

4.8.4. **RFLP of Bacterial 16SrDNA**

To analyse the genetic variation between the bacterial isolates (UAB 5, 16, 18, 19) restriction fragment length polymorphism (RFLP) was implemented so as to rule out possible identical bacterial isolates. All the restriction enzymes chosen for this technique were tetra-cutters which were used to create large number of fragments as number of cleavage depends upon the number of restriction sites present in the fragment. The agarose gel electrophoresis was the final step to visualise the fragments of DNA in form of bands as shown in figure (4.13). The size of fragments were different when cut with Taq I restriction enzyme, which reflected the dissimilarity between 2 bacterial isolates (represented in Lane 9) and similarity between rest of two is assumed due to restriction digestion by Alu I and Hpa II. The analysis declared the similarity between UAB 9, UAB16 and UAB 18 bacterial isolates while it is different from UAB 5 strain.

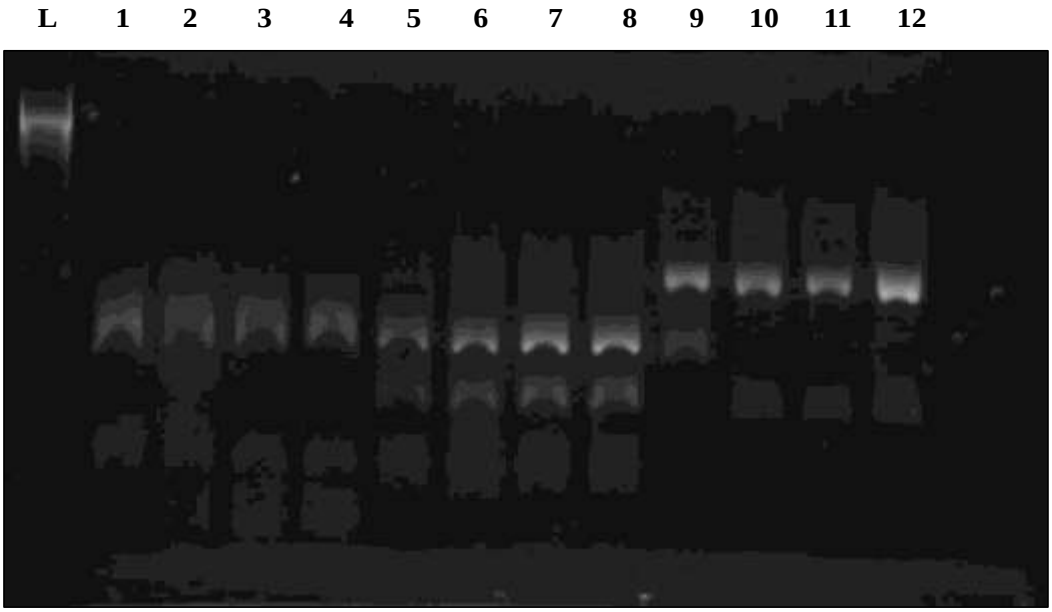

Figure 4.13: Distinct bands of RFLP for each DNA isolate restricted with different restriction enzyme. Lane 1-12: 1- UAB 5 (Alu I), 2- UAB 9 (Alu I), 3- UAB 16 (Alu I), 4- UAB 18 (Alu I), 5- UAB 5 (Hpa II), 6- UAB 9 (Hpa II), 7- UAB 16 (Hpa II), 8- UAB 18 (Hpa II), 9- UAB 5 (Taq I), 10- UAB 9 (Taq II), 11-UAB 16 (Taq I), 12-UAB 19 (Taq I), L: ladder.

4.8.5. Genetic transformation and blue-white screening

As inferred from RFLP analysis, 2 distinct isolates were chosen for their identification. Ligation of their 16S rDNA was performed by TA cloning. This method utilizes a T-vector pMD20T, a plasmid vector with single deoxyribothymidine (dT) addition at its 3' end and this complementarity between dT overhang and dA overhang of PCR product results in

cloning. This cloned product was transformed in *E. coli* DH10ß competent cells. To ensure successful ligation in plasmid vector, transformed competent cells were grown in selective media supplemented with X-gal and IPTG. IPTG is a lactose metabolite that does not gets hydrolysed by ß-galactosidase and prevents the cell from degrading the inducer while X-gal tests for the presence of ß- galactosidase enzyme and this enzyme-catalysed hydrolysis yields insoluble blue coloured compound represented as blue colonies. Figure (4.14) represented the Blue and white colonies of non-transformed and transformed products respectively. Random white colonies were picked for isolation of their plasmid DNA. Colony PCR confirmed the presence of insert DNA in plasmid construct.

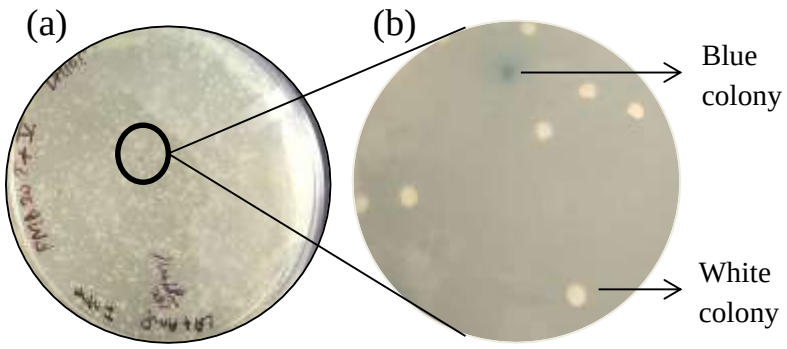

Figure 4.14: (a) Blue-white screening on LA+ ampicillin plates (b) Distinct blue and white colonies as seen in LA+Ampicillin plates

4.8.6. Plasmid DNA isolation and insert amplification

Plasmid DNA when subjected to PCR amplification by M_{13} primers (figure 4.5) and analysed using agarose gel electrophoresis confirmed the presence of inserts of size 1.5 kb. The bands were observed to be of 1.5 kb as compared to ladder sequence.

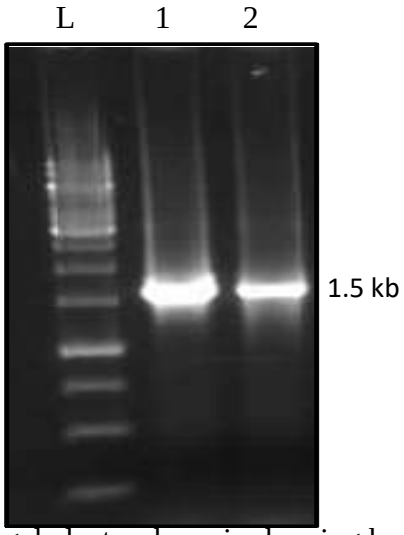

Figure 4.15: Agarose gel electrophoresis showing bands of plasmid DNA in bacterial isolates Lane 1-2: 1: UAB 5, 2: UAB 16, L: Ladder.

4.8.7. 16S rDNA Sequenced regions:

Sequencing of selected clones of UAB 5 and UAB 16 isolates was performed using M13-F and M13-R primers. Obtained sequences for both the isolates are mentioned as follow:

>UAB5

TACGGCTACCTTGTTACGACTTCACCCCAATCATCTGTCCCACCTTAGGCGGCTAGCTCCTTACGGTT
ACTCCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACG
TATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCATGTAGGCGAGTTGCAGCCTA
CAATCCGAACTGAGAATGGTTTTATGGGATTGGCTTGACCTCGCGGTCTTGCAGCCCTTTGTACCATC
CATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCC
GGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTAAATGCTGGCAACTAAGATCAAGGGTTGCGCT
CGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTG
TCCCCGAAGGGGAACGCTCTATCTCTAGAGTTGTCAGAGGATGTCAAGACCTGGTAAAGGTTCTTCGC
GTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCCTTTGAGTTTCAG
TCTTGCGACCGTACTCCCCCAGGCGGAGTGGCTTAATGCGTTAGCTGCAGCCACAGGCGGAGTGCTTA
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GCCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCCGCTTT
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GCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTG
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CCTATGAAGGAGAAGATCCTATCCGGTATTAGCTTCGGTTTCCCGAAGTTATCCCAGTCTTACAGGCA
GGTTGCCCACGTGTTACTCACCCGTCCGCCGCTAACGTCATAGAAGCAAGCTTCTAATCAGTTCGCTC
GACTTGCATGTATTAGGCACGCCGCCAGCGTTCATCCTGAGCCAGGATCAAACTCTAAT

>UAB16

TGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACAGAT
AAGGAGCTTGCTCCTTTGACGTTAGCGGCGGACGGGTGAGTAACACGTGGATAACCTACCTATAAGAC
TGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAACATATTGAACCGCATGGTTCAATAGTGAAA
GGCGGCTTTGCTGTCACTTATAGATGGATCCGCGCCGTATTAGCTAGTTGGTAAGGTAACGGCTTACC
AAGGCAACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACT
CCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAG
TGATGAAGGTCTTCGGATCGTAAAACTCTGTTATCAGGGAAGAACAAATGTGTAAGTAACTGTGCACA
TCTTGACGGTACCTGATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGG
CAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCC
CACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCC
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CGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCC
TGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATG
TGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTTTGACCGCTCTAGAGATAG
AGTCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGT
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TGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGG
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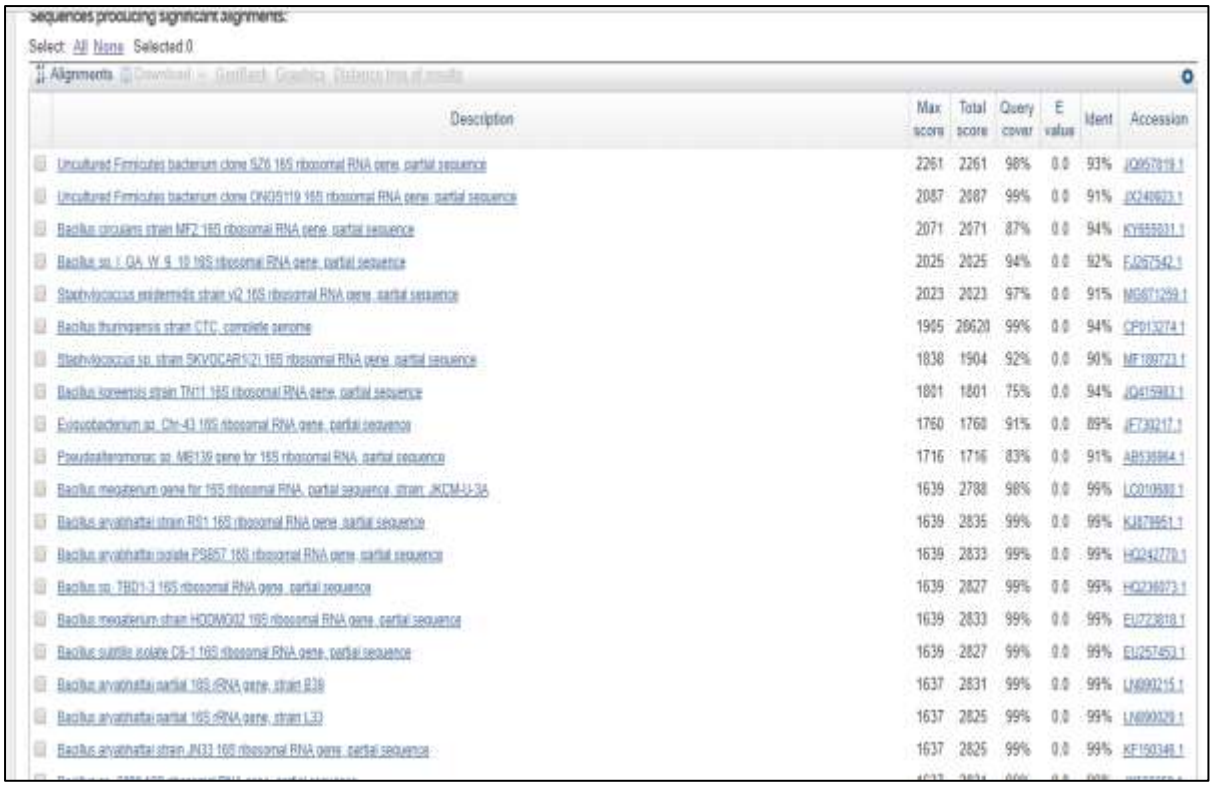
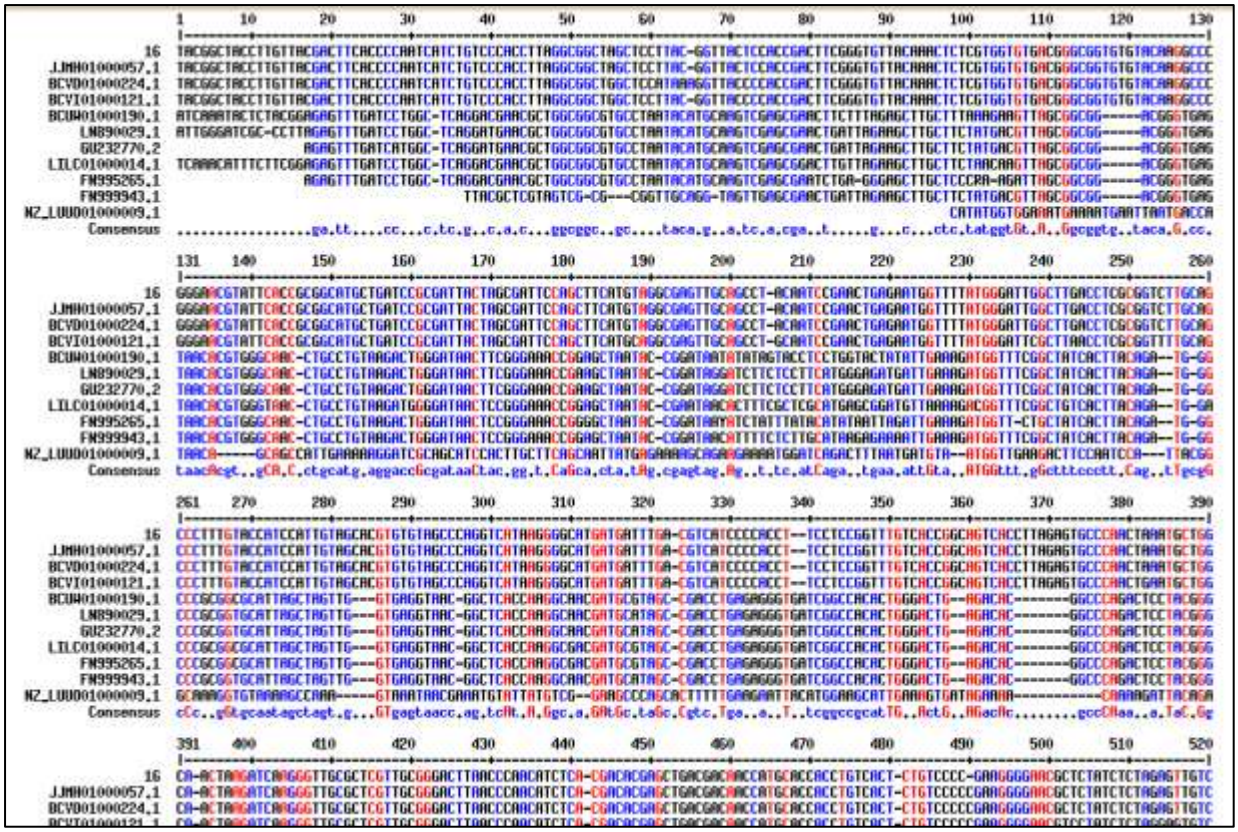
TTGTTCTCAGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGTAGAT
CAGCATGCTACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAA
CACCCGAAGCCGGTGGAGTAACCATTTATGGAGCTAGCCGTCGAAGGTGGGACAAA

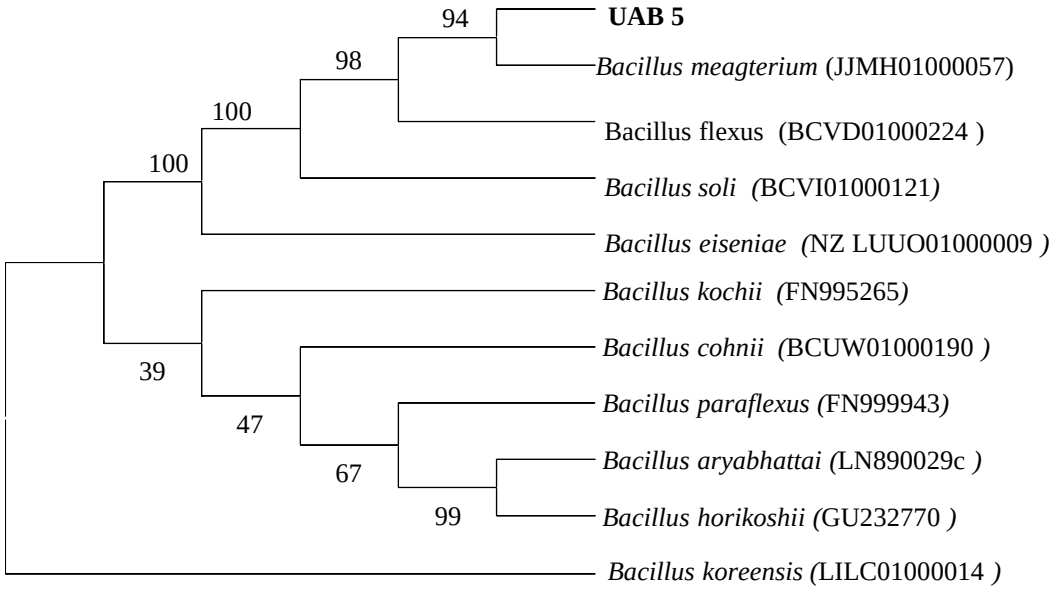
The phylogenetic trees were constructed with sequences of both bacterial isolates obtained by BLASTN. Multiple sequence alignment using multalin interface provided progressive pair wise alignment of related group of sequences. The query sequence was aligned with generated blast sequences as shown in figure (4.16). The phylogenetic tree was created using Mega 7 software. The history of evolution of the query sequence was evaluated using maximum parsimony method. The evolutionary history was inferred using the maximum parsimony method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches.



(a)

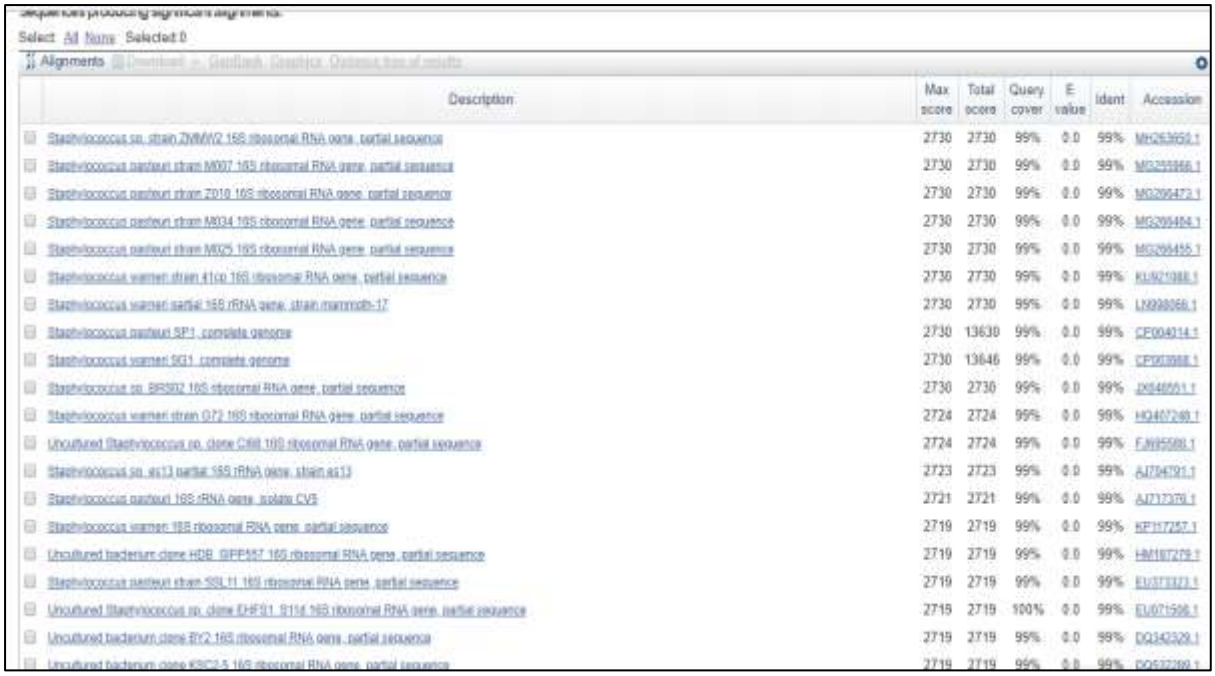


(b)



(c)

Figure 4.16: Phylogenetic relatedness of bacterial isolate *Bacillus megaterium* (UAB 5) (a) Blast analysis, (b) multiple sequence alignmnent using multalin (c) Maximum parsimony tree bassed on different isolates of current study.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Accession
Staphylococcus sp. strain ZNMW2 16S ribosomal RNA gene, partial sequence 2730 2730 99% 0.0 99% MH263650.1
Staphylococcus pasteuri strain M007 16S ribosomal RNA gene, partial sequence 2730 2730 99% 0.0 99% MG255966.1
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Staphylococcus warneri SG1, complete genome 2730 13646 99% 0.0 99% CP000988.1
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Staphylococcus warneri strain G72 16S ribosomal RNA gene, partial sequence 2724 2724 99% 0.0 99% HQ407249.1
Uncultured Staphylococcus sp. clone C468 16S ribosomal RNA gene, partial sequence 2724 2724 99% 0.0 99% FJ695588.1
Staphylococcus sp. as13 partial 16S rRNA gene, strain as13 2723 2723 99% 0.0 99% AJ784791.1
Staphylococcus pasteuri 16S rRNA gene, isolate CVS 2721 2721 99% 0.0 99% AJ717379.1
Staphylococcus warneri 16S ribosomal RNA gene, partial sequence 2719 2719 99% 0.0 99% KP117257.1
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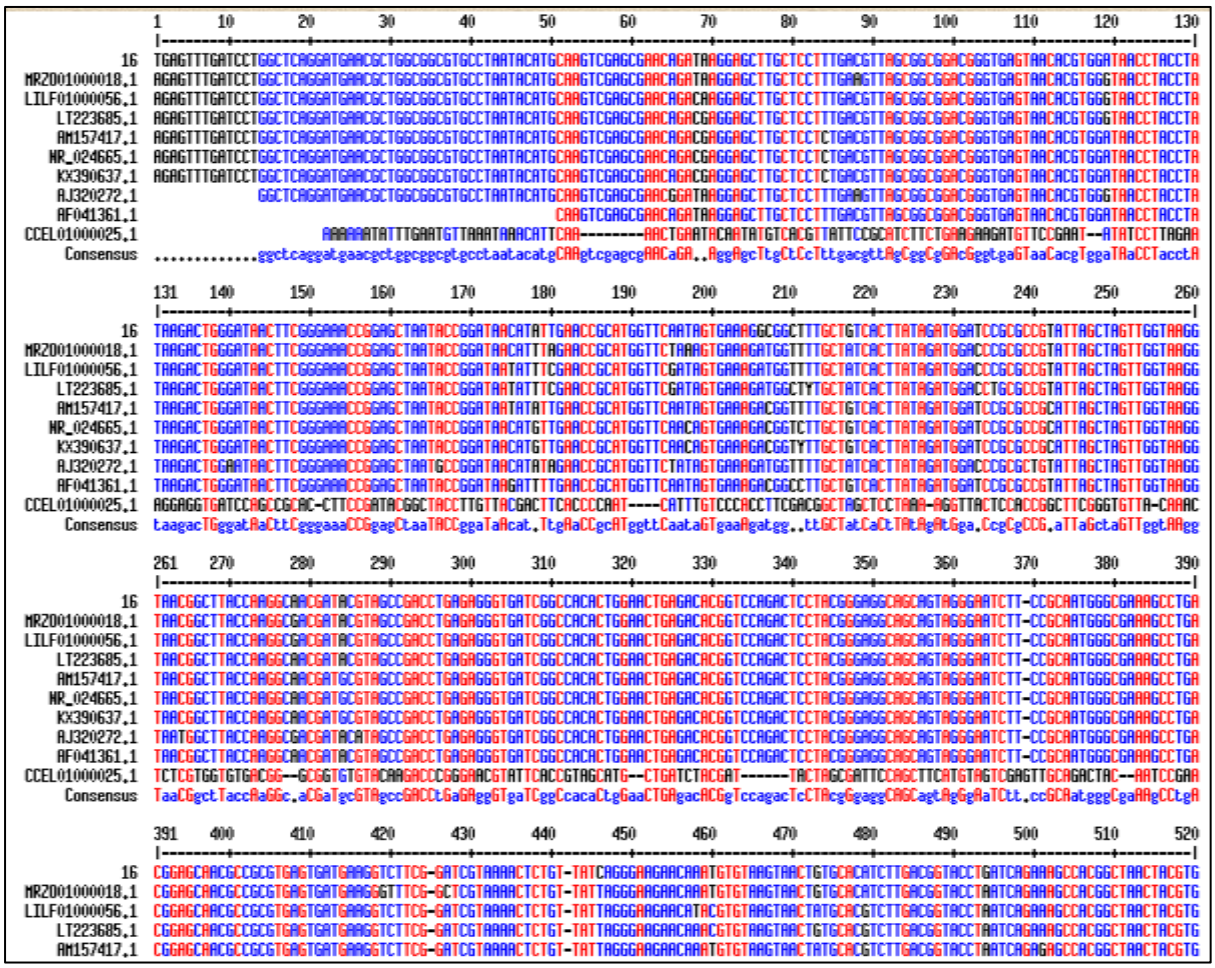
(a)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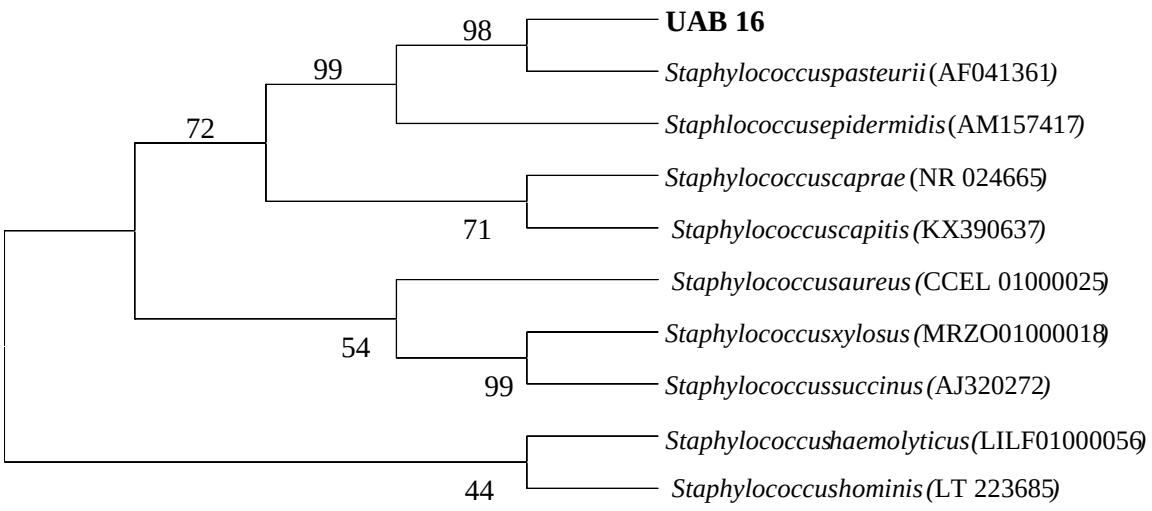
Consensus

(b)

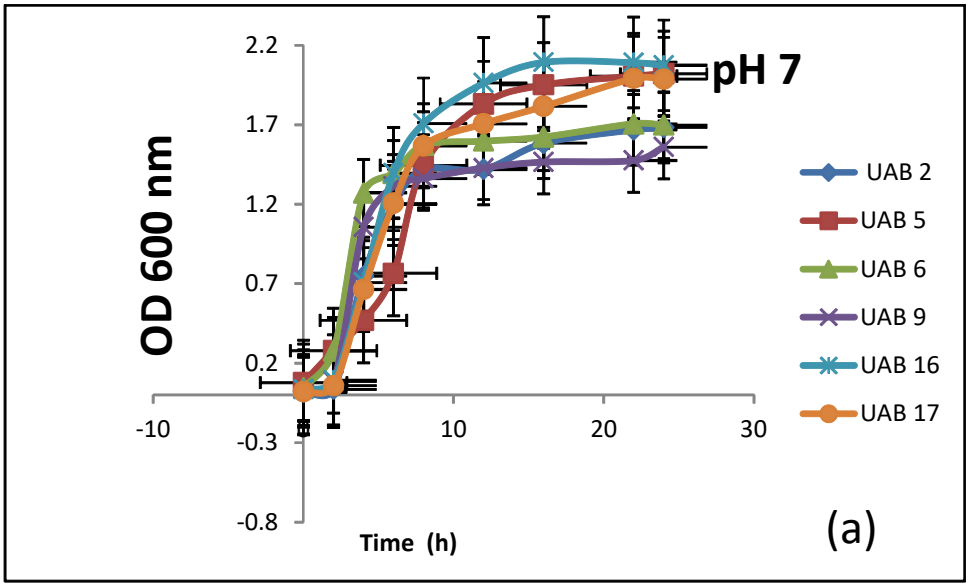


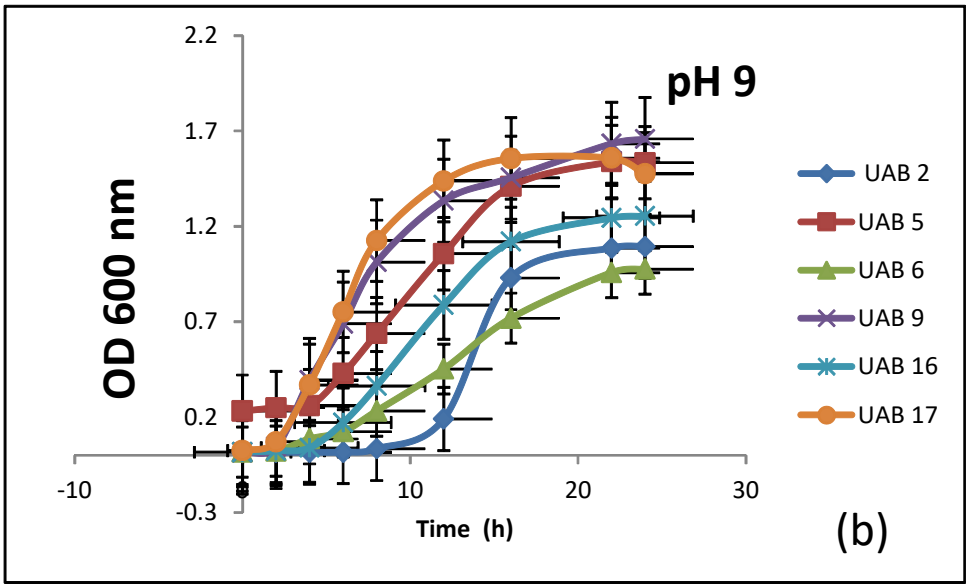
(c)

Figure 4.17: Phylogenetic relatedness of bacterial isolate *Staphylococcus pasteurii* (UAB 5); (a) Blast analysis, (b) multiple sequence alignmnent using multalin (c) Maximum parsimony tree based on different isolates of current study.

4.9. Physiological Characterization

Conditions of high pH were provided to bacterial isolates to analyse their survival in highly alkaline conditions. This was performed for a period of 24 hours. The growth of selected isolates i.e., *Bacillus megaterium* (UAB 5) and *Staphylococcus pasteurii* (UAB 16) was compared with previously isolated bacterial samples at different pH conditions i.e., 9, 10, 11. This analysis between various isolates has been graphically represented in figure 4.18. The growth continued to occur up to 20 hours at pH 9 and then arrives at stationary phase. Similar kind of behaviour was observed for all isolates at pH 7 but kinetics was best studied in both bacterial isolates.


pH 7
OD 600 nm
2.2
1.7
1.2
0.7
0.2
-0.3
-0.8
-10
10
20
30
Time (h)
UAB 2
UAB 5
UAB 6
UAB 9
UAB 16
UAB 17
(a)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OD 600 nm
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1.7
1.2
0.7
0.2
-0.3
-10
10
20
30
Time (h)
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UAB 5
UAB 6
UAB 9
UAB 16
UAB 17
(b)

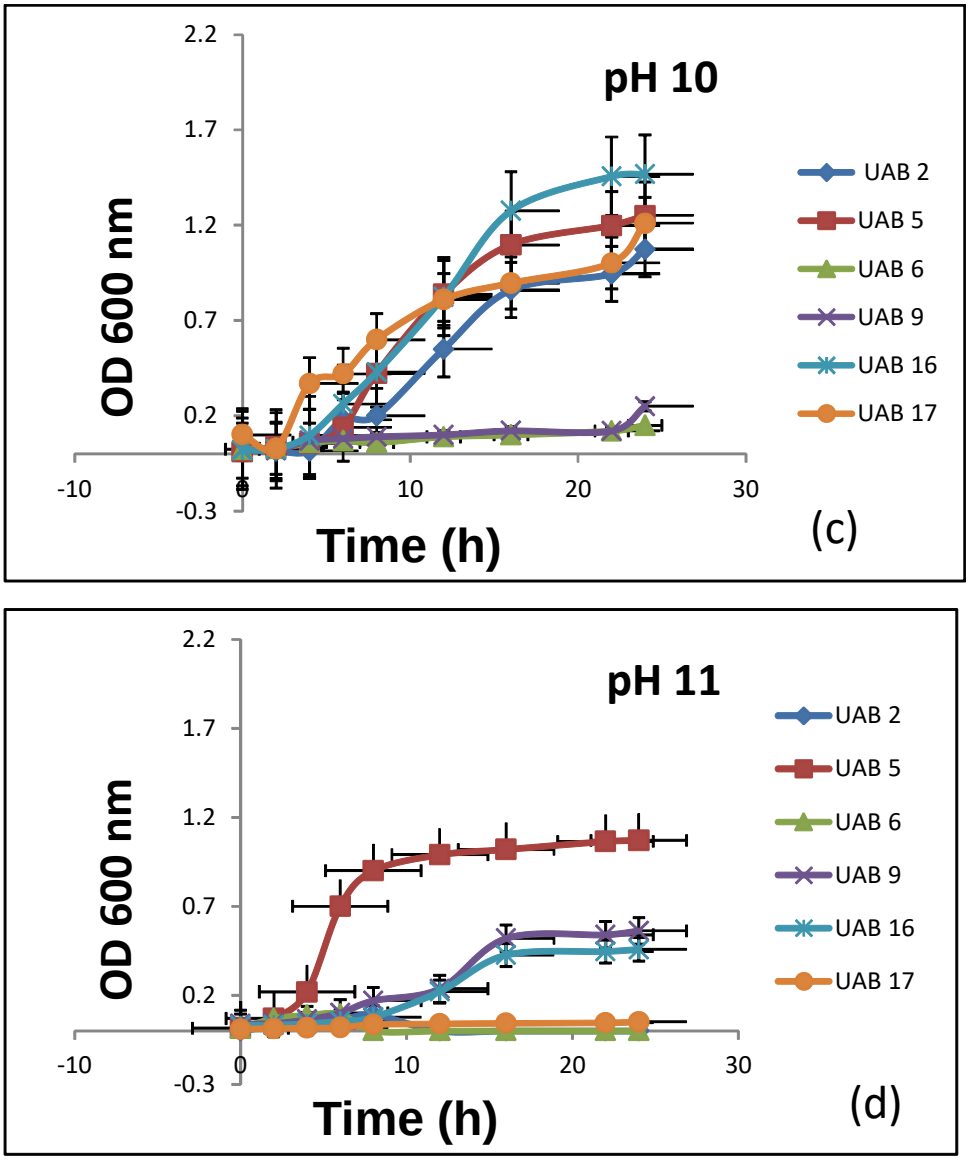


Figure 4.18: Growth curve showing survival rate of bacterial isolates at different alkaline conditions (a) at pH 7, (b) at pH 9, (c) at pH 10 and (d) at pH 11.

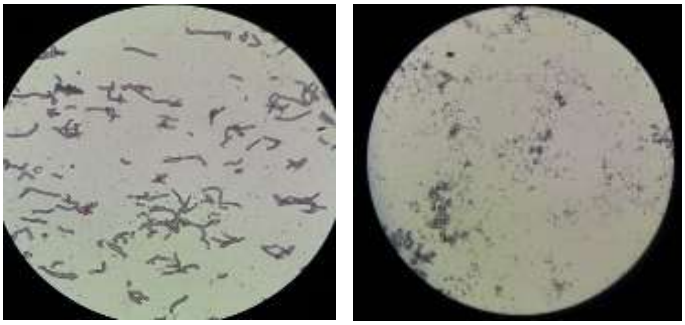
4.10. Morphological and Biochemical Characterization:

Both the bacterial isolates i.e., UAB 5 and UAB 16 were exposed to various morphological and biochemical characterizations. The Gram character (Figure) of both the isolates was found to be Gram positive. UAB 5 was found to be rod shaped (in chains), UAB 16 showed cocci (present singly) while both appeared as opaque – creamy colonies. No pigmentation was visible in colonies of both isolates when grown on nutrient media. Positive or negative reactions were best observed using pictorial representation as shown in figure (4.19).

Table 4.3: Biochemical characterizations of bacterial isolates

BIOCHEMICAL REACTIONS	UAB 5	UAB 16
Gram staining		
Stain	+	+
Morphology	**Rods**	**Cocci**
Chain/single	**Chains**	**Groups**
Motility	+	-
Oxidase	+	-
Catalase	+	+
Nitrate Production	-	+
Urease test	+	+
Sugar fermentation test		
Glucose	+	+
Lactose	-	-
Mannitol	-	-
Mannose	-	-
Gas-production during sugar fermentation		
Glucose	-	-
Lactose	-	-
Mannitol	-	-
Mannose	-	-
Hi-aureus coagulase confirmation test	-	-
Mannitol Salt agar test	-	+

+ : Positive reaction, ⁻ : Negative reaction



(a) 5 (b) 16

Figure 4.19: Gram staining representing colony morphology

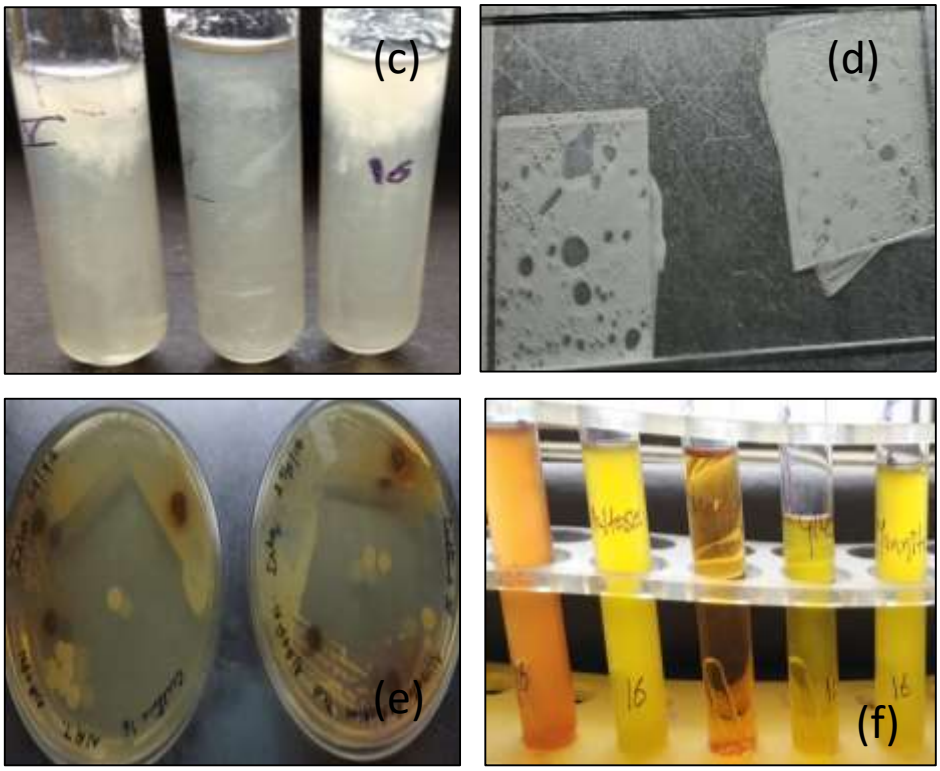


Figure 4.20: Pictorial representation of biochemical tests (c) Motility test (UAB 5, control, UAB 16), (d) Catalase test (UAB 5, UAB 16), (e) Nirate reduction test (UAB 5, UAB 16), (f) Carbohydrate fermentation test (UAB 16)

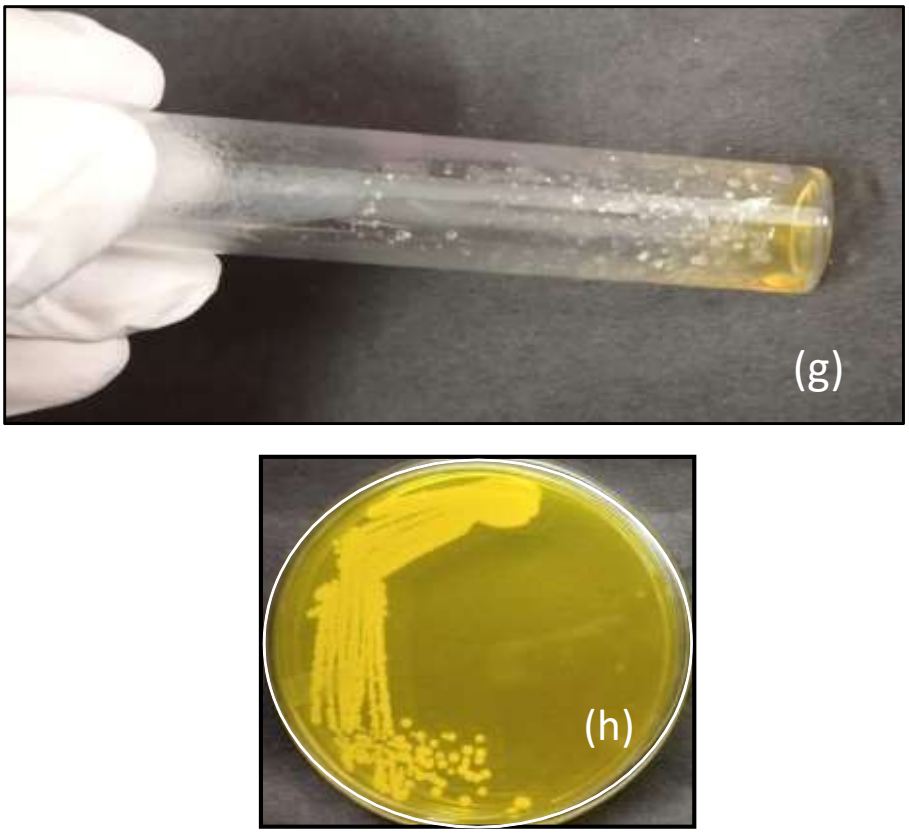


Figure 4.21: Biochemical characteristics of bacterial isolates (g) Hiaureus coagulase confirmation test (UAB 16), (h) Mannitol salt agar test (UAB 16)

4.11. Evaluation of microbes for enhancement of strength in mortar cubes

4.11.1. Selection of strain:

The concept of biomineralization was to precipitate calcium carbonate in pores of mortar by use of microbes. Presence of urease can only cause degradation of urea and ultimately precipitation of calcium carbonate. Thus ureolytic bacteria could be exploited as source of urease so as to perform urease activity and calcite precipitation in alkaline environments of mortar and cement materials. So, present work has been focussed on calcium carbonate precipitation by means of bacterial enzymatic activity. The bacterial isolate chosen was *Bacillus megaterium* (UAB 5) showing highest urease and calcite precipitation activity and growth kinetics at different pH concentrations demonstrated efficient growth of this bacterial isolate as compared to *Staphylococcus pasteurii* (UAB 16) and also other isolated bacterial strains from steel slag sample.

4.11.2. Initial and final setting time of cement pastes

Use of slag powder as replacement with cement was done to study its impact on initial and final setting time of cement paste. The initial and final setting time (figure 4.22) of control mix was observed to be 120 minutes and 200 minutes respectively while it was also recorded for cement slag mixes. The graphical representation concluded that 50% incorporated cement slag mix showed highest increase in final setting time while not much rise in initial setting time was observed as compared to control mix. Cement slag specimens with 20%, 30% and 40% replacement showed minor increase in final setting time as compared to control specimens while initial setting time remained comparatively similar.

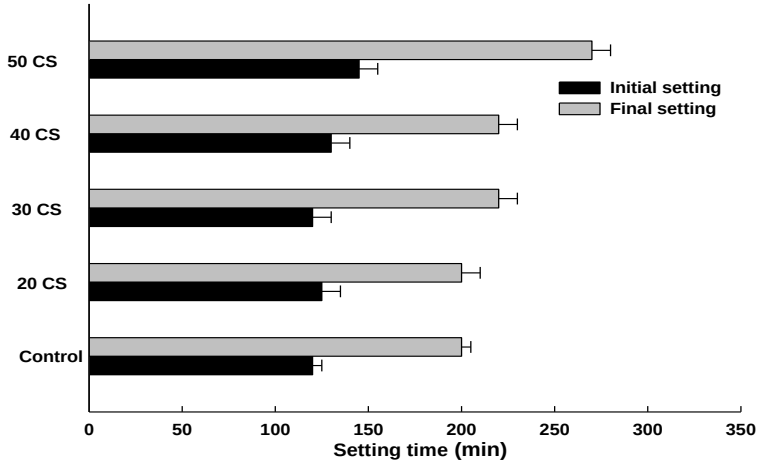


Figure 4.22: Variation in Initial and final setting time of cement slag paste as compared to control

4.11.3. Compressive strength of mortar cubes:

Visual observations in figure (4.2) indicated difference in surface morphology of cement slag (CS) specimens (at different ratios) as compared to control specimen. In case of cement slag bacterial treated (CSBT) specimens, curing with *Bacillus megaterium* was done for 28 days to study the compressive strength of the specimens. As the specimens were submerged in bacterial culture, urea and $CaCl_2$ solution, calcium carbonate precipitation occurred at the surface or near surface regions of mortar specimens. SEM-EDX analysis of specimens, as depicted in figure (4.23), showed calcium carbonate crystals in between silica crystals and were compared with control specimen. The presence of $CaCO_3$ was confirmed by the peaks in EDX graphs. Rhombohedral calcite crystals and sphaerical shaped vaterite crystals were present in cement slag bacterial treated (CSBT) specimens.

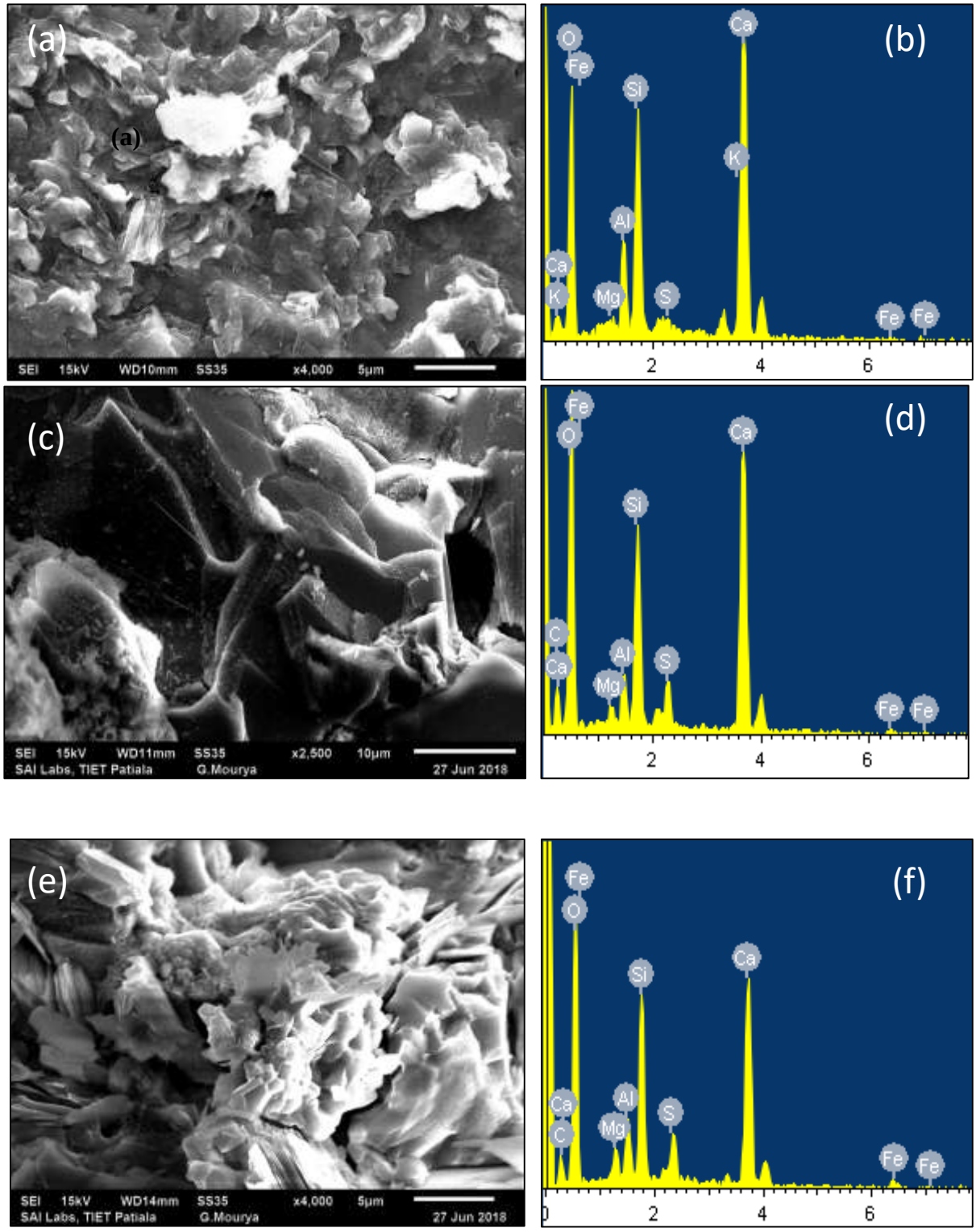


Figure 4.23: SEM-EDX images of cement slag (CS) specimens (a),(b) control specimen, (c),(d) CS-20, (e),(f) CS-30

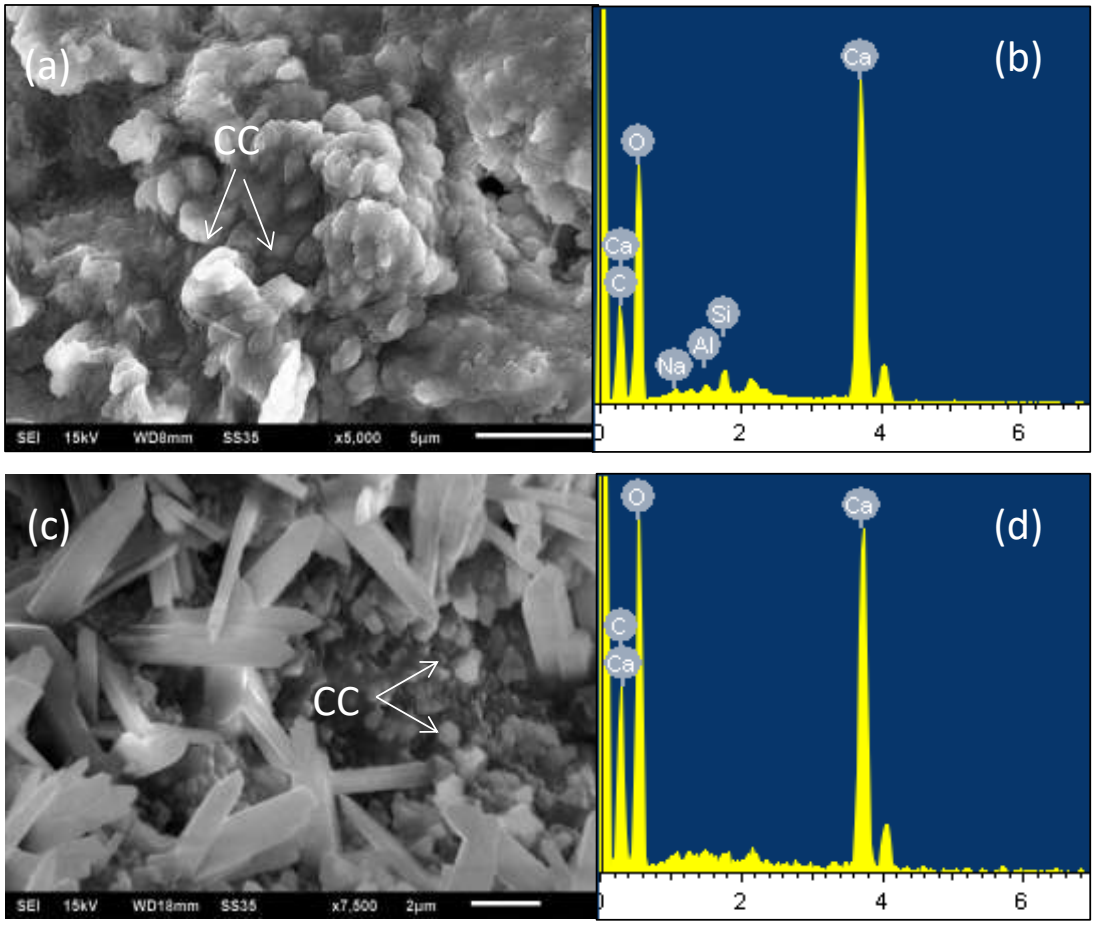


Figure 4.24: SEM-EDX images represent calcium carbonate crystals in cement slag bacterial treated (CSBT) specimens (a),(b) CSBT-20 (c),(d) CSBT -30

Apart from microstructural analysis, compressive strength was measured in Mpa figure (4.25). A 5% increase was observed in 20% CSBT specimen with as compared to control specimen while 30% replaced CSBT specimen showed comparable compressive strength with control specimen. It was observed that 40% and 50% CSBT specimens showed 5-6% decreased strength as compared control specimen but compressive strength increased by 3-4% when compared to CS (cement slag specimens).

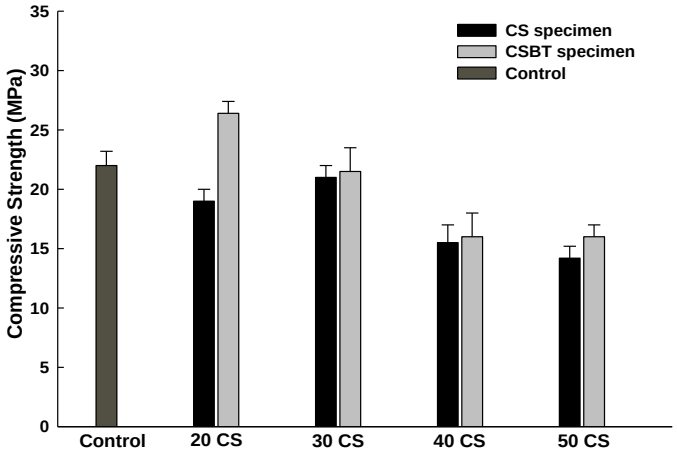


Figure 4.25: Compressive strength of CS and CSBT specimens at age of 28 days curing.

4.11.4. Water absorption test:

The decrease in permeability of mortar specimens treated with bacteria was analysed through water absorption test. Figure (4.26) showed influence of slag in cement slag mortar cubes and also bacterial surface treatment on cement slag bacterial treated specimens. 20% and 30% CSBT specimens showed nearly 6 times less water absorption as compared to control specimens. 40% and 50% CSBT specimens showed 7-8 times less absorption of water as compared to control specimens.

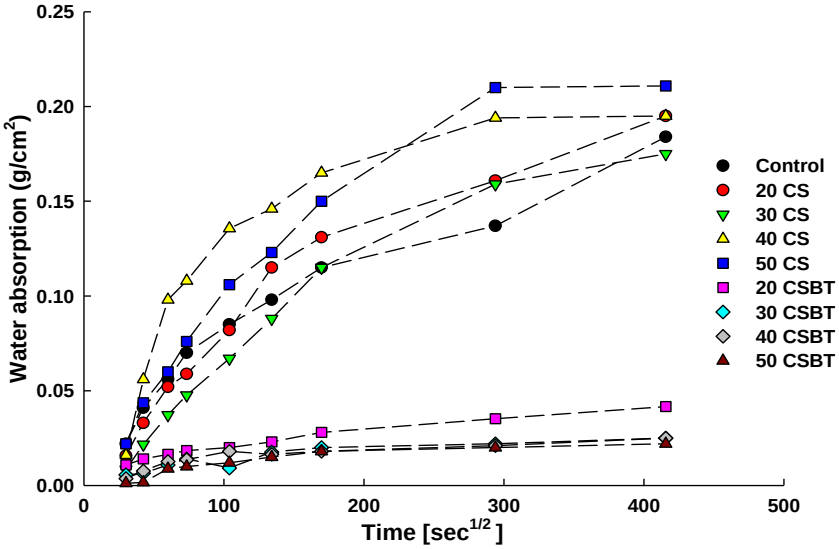


Figure 4.26: Influence of CS and CSBT specimens on water absorption rate with effect of time

DISCUSSION

Production of microbial mortar required ureolytic alkaline bacteria which can easily adapt in alkaline conditions. These bacteria are known to cause calcium carbonate precipitation in presence of urease enzyme and it also metabolizes in alkaline environment thus steel slag was utilized as source for bacterial isolation. Reportedly, for the survival of bacteria at this pH certain structural components of cell wall may contribute to pH homeostasis as reported by Aono et al (1999). Andrews et al (1995) also determined ureolytic activity of bacterial isolates by their utilization of urea and degradation of phenol red. After the determination of their ureolytic potential, non-homologous isolates were selected by analysing their restriction fragment length polymorphism. These distinct isolates were only subjected to genetic operations and genetic variability was determined by means of sequencing of their 16S rDNA. The phylogenetic analysis revealed UAB 5 and UAB 16 to be *Bacillus megaterium* and *Staphylococcus pasteurii* isolates respectively. According to Achal et al (2011), Bacillus strain were believed to undergo calcium carbonate precipitation and were further used to investigate compressive strength and water absorption of mortar structures. *Bacillus megaterium* (UAB 5), due to high calcite precipitation activity and urease activity analysis, was exploited to investigate its effect on strength properties of mortar cubes. Cement slag bacterial treated specimens with 20% and 30% replacement revealed nearly 6 times less water absorption as compared to control specimens. Reduction in water absorption in fly ash amended mortar specimens was also reported by Achal et al (2011). Presence of bacteria was believed to decrease the water uptake rate in mortar specimens and thus enhanced the properties of mortar specimens. Treatment of mortar specimens with bacillus sp. is reported by Joshi et al (2018) to enhance the compressive strength after 28 days as compared to control. This study also inferred 5% increased compressive strength of cement slag bacterial treated specimens at 20% replacement and comparable increase in 30% replacement. 5-6% decrease was observed in 40-50% replacement levels of CSBT specimens along with 7-8 times less water absorption in similar replacement levels. Decrease in compressive strength was also reported to occur due to certain factors as discussed by Ersan et al (2015). Thus the use of this strain can be considered effective to be utilized for surface treatment of mortar specimens with lower levels of slag incorporation.

CONCLUSIONS

The Microbially induced calcium carbonate precipitation has been exploited in this present study so as to potentially enhance the durability or strength of cementitious materials. The replacement of cement in mortar specimens was being carried out to greatly reduce the use of cement to make it eco-friendly and sustainable building material. Also, replacement done with industrial by-product, steel slag, caused reduction in environment pollution and was possibly believed that it is having similar or more potentials than cement. Treatment of cement slag bacterial treated specimens with bacteria *Bacillus megaterium* isolated from the steel slag showed calcium carbonate precipitates. Amendment of slag powder in mortar cubes showed more resistance towards water absorption which is a major factor to reflect the benefit of replacement of cement with slag in mortar specimens. The cement slag bacterial treated specimens showed decreased water absorption levels as compared to cement slag mortar specimen. The microbial treatment resulted in increased compressive strength of mortar cubes at low replacement levels as compared to control specimens but it also increased as compared cement slag specimens Not much effective strength changes were observed cement slag bacterial treated specimens with high replacement levels as compared to control. Thus low replacement slag levels can be amended in mortar materials. 5% decreased compressive strengths along with six times less water absorption can be considered as a factor for the further studying the use of mortar specimens with low levels of slag replacement and use of cement slag bacterial treated specimens can be potentially exploited and modified to achieve enhanced results.

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APPENDIX I

1X buffer Taq I

Tris HCl (pH 8.0)	10 mM
$MgCl_2$	5 Mm
NaCl	100 mM
BSA	0.1 mg/ml

1X Tango buffer

Tris Acetate (pH 7.9)	33 mM
Magnesium acetate	10 mM
Potassium acetate	66 mM
BSA	0.1 mg/ml

$CaCl_2$ (0.1M)

$CaCl_2$ dihydrate	1.47 g
Distilled water	100 ml

Calcium chloride ($CaCl_2$) (25 mM)

$CaCl_2$ dihydrate	0.367 g
Distilled H_2O	100 ml

Fermentation Medium

Peptone	10.00
Carbohydrates	5.00
Sodium chloride	15.00
Phenol red	0.018
pH	7.3

Glycerol stock (80%)

100% glycerol	80 ml
Distilled water	20 ml

Luria broth (HiMedia, India)

Caesin enzymic hydrolysates	10.00
Yeast extract	5.00
Sodium chloride	5.00

pH	7.0±0.2

Lysozyme solution

lysozyme crystals	10 mg
Distilled H_2O	1 ml

Nutrient agar (HiMedia, India):

Casein hydrolysates	15.0
Peptone	5.0
NaCl	5.0
pH	7.5

Nutrient Broth (HiMedia, India)

Casein hydrolysates	15.0
Peptone	5.0
NaCl	5.0
pH	7.5

Potassium Phosphate Buffer (0.1 M)

Monobasic dihydrogen phosphate salt (K_2HPO_4)(0.1 M)	1.741 g
Dibasic monohydrogen phosphate salt (KH_2PO_4)(0.1 M)	1.36 g
Distilled H_2O	1000 ml

9.4 ml K_2HPO_4 + 0.6 ml KH_2PO_4 + 90 ml Distilled H_2O

Saline-EDTA (0.5 M)

EDTA	18.6 g
NaOH pellets	
pH	8.0
NaCl (0.15 M)	0.85 g
Distilled H_2O	100 ml

Solution I (DNA isolation)

Glucose	50 mM
EDTA	10 mM
Tris-HCl	25mM

Solution II

NaOH	0.2 M
SDS	1%

Solution III

Potassium acetate (5 mM)	60 ml
Glacial acetic acid	11.5 ml
Distilled water	28.5 ml

TBE Buffer (10 X)

Tris-HCl	0.09 M (pH 8)
Boric acid	0.9 M
EDTA	0.02 M (pH 8)

Urea solution (2%)

Urea	20 g
Distilled water	1000 ml

Urea (40%)

Urea	40 g
Distilled H_2O	100 ml

Urea agar base medium (HiMedia, India)

Gelatin peptone	1.0
Dextrose	10
Potassium phosphate	2.0
NaCl	5.0
Phenol red	12.0 mg/l
Urea	20.0
Agar	20.0
pH	7.5