Biomineralization by bacteria - as a waste management technique

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ABSTRACT

Cement is already the most used man-made material in the world as it is relatively cheap and its basic ingredients (sand/ gravel/ water) are readily available.Even though there has been increased inquisitiveness for Bio-cement but is clear that cement, the key binder ingredient in that is of a high environmental impact. Today innovation is leadingly being inspired by nature as a sustainable alternative. Hence, taking notes from biomimicry and biotechnology, investigation is being conducted to create concrete the way nature does with microorganisms. My work here I've tried to explore a sustainable design application involving biological treatment of sand thorough microbially-induced calcite precipitation (MICP), which produces minerals by bacterial metabolic activity. Since most of the studies on MICP thus far have focused on limited fields such as engineering, biotechnology, and geo-technology, this study has focused more on improving the application of bio-cement and reducing its carbon footprint by controlled assessment other detrimental biotic and abiotic factors.I worked on implementation of producing a Biocementation brick with a combination of nonbiological/chemical sources like epoxy which sure would improve its mechanical properties. S. Pasteurii and its role in MICP are topics of active research and several issues relating to the mechanism of chemical precipitation are still not fully understood. The sub-principles of this study were to review the possibilities of having the positive and negative impacts of bio-concrete application in the aspects of strength, durability,affect on human health etcetra and carrying out a theoretical review about the same . I wanted to address that at present there isn't any suitable /established Biocementation method for large-scale application .Moreover I want to study the Mechanical characterization of Bio-calcified sand plus the aesthetic and scalability study of bio-cement.This study can to conduct new research to find a solution for decreasing of negative impacts and increasing positive impacts of biological concrete to make it more trustworthy.

Keywords: Bio-concrete, MICP, Life-Cycle-Assessment (LCA)

Glossary

Biomimicry The mimicking of life using imitation biological systems

Urease An enzyme that catalyses the hydrolysis of urea to form ammonium carbonate

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1. INTRODUCTION

1.1. Background

Calcite (CaCO3) is one of the most common and widespread minerals on Earth, constituting 4% by weight of the Earth's crust. It is naturally found in extensive sedimentary rock masses, as limestone, marble and calcareous sandstones in marine, freshwater and terrestrial environments. The oldest known surviving concrete as derivative of calcites is found in the former Yugoslavia and is thought to have been laid in 5600 BC using red lime as the cement. The first major concrete users were the Egyptians around 2500 BC; Egyptians used mud mixed with straw to bind dried bricks. Later the Romans since 300 BC made many developments in concrete technology including the use slaked lime a volcanic ash called pozzuolana; animal fat, milk, and blood were used as admixtures. As engineers struggle with the difficult task of rehabilitating deteriorating infrastructure, there may be lessons to learn from the satisfactory long-term performance of ancient concrete structures.These are the structures that have been there sturdy for almost a millennia with only minor repairs which makes us retrospect that there is some phenomenon which titillates one's school of thought and may have been there all along but whole human race failed in the face of necessity

1.2 **Biomineralization and MICP(Microbiologically Induced CaCO3/Calcite Precipitation)**

Biomineralization is the chemical alteration of an environment by microbial activity that results in the precipitation of minerals (Stocks-Fischer et al. 1999; Barkay and Schaefer 2001; Phillips et al. 2013). In nature, biomineralization is a widespread phenomenon leading to the formation of more than 60 Million different biological minerals (Sarikaya 1999) that exists as extracellularly inorganic crystals (Dhami et al. 2013a) or intracellularly (Konishi et al. 2006; Yoshida et al. 2010). Extracellular mineralization syntheses (for e.g., carbonate precipitation) from all groups of living organisms are widespread and well known phenomena (Lowenstam 1981). Most crystals formed through biomineralization consist of inorganic minerals, but they may also contain trace elements of organic compounds, which can regulate the biomineralization process (Yoshida et al. 2010). There are three different mechanisms involved in the production of biominerals: (1) Biologically controlled mineralization consists of cellular activities that specifically direct the formation of minerals (Lowenstam and Weiner 1989; Benzerara et al. 2011; Phillips et al. 2013).

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

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

Microbial carbonate precipitation (MCP) has gained interest in the last 20 years, particularly with regard to the potential role marine systems may play as 'carbon sinks' for the increasing global production of $CO2$.

Three main groups of organisms exist that can induce MCP through their metabolic processes;

(i) Photosynthetic organisms such as cyanobacteria and algae that remove CO2,
(ii) Sulphate reducing bacteria that are responsible for the dissimilatory reduction of sulphate and

(iii) Several organisms that are involved in the nitrogen cycle (Castanier et al., 1999; Hammes and Verstraete, 2002).

The most common form of MCP in aquatic environments is caused by photosynthetic organisms (McConnaughey and Whelan, 1997). The metabolic processes of algae and cyanobacteria utilize dissolved $CO2$ (Eqn 1), which is in equilibrium with $HCO3-\ and\ CO32-$ (Eqn 2). The removal of $CO2$ induces a shift in this equilibrium, and results in an increase in pH (Eqn 3) .When this reaction occurs in the presence of calcium ions, calcium carbonate is produced (Eqn 4) (Hammes and Verstraete, 2002).

$$CO_2 + H_2O \rightarrow CH_2O + O_2 \quad (1)$$

$$2HCO_3^{2-} \rightarrow CO_2 + CO_3^{2-} + H_2O \quad (2)$$

$$CO_3^{2-} + H_2O \rightarrow HCO_3^- + OH^- \quad (3)$$

$$Ca^{2+} + HCO_3^- + OH^- \rightarrow CaCO_3 + 2H_2O \quad (4)$$

Calcite can also be precipitated by heterotrophic organisms, by the production of carbonate or bicarbonate and modification of the environment to favour precipitation (Castanier et al., 1999).

MICP can also be induced by organisms involved in the nitrogen cycle, via ammonification of amino acids, nitrate reduction and the hydrolysis of urea. The simplest of all of the mechanisms described for MICP is the hydrolysis of urea by the enzyme urease, which results in the production of carbonate ions in the presence of ammonium (Eqn 5). Calcite is readily precipitated under these conditions, in the presence of calcium.

$$CO(NH_2)_2 + 2H_2O \rightarrow CO_3^{2-} + 2NH_4^+ \quad (5)$$

1.2.1 *Factors affecting the efficiency of MICP:*

1. Type of bacteria
2. Bacterial cell concentration
3. pH
4. Temperature
5. Urea and Ca2+concerntration

1.3 **Envisage the complications of using Bio-cement.**

It has been clear that cement, the key binder ingredient in concrete has a high environmental impact. Presently about 10% of the total anthropogenic CO_2 is due to the cement production solely **(**Jonkers 2009**)**. The main concern is that concrete is unsustainable due to the extensive carbon footprint associated to it.The use of bio-concrete significantly influences the strength of concrete by making its permeability lower than conventional concrete and at the same time, offering great resistance to freeze-thaw attacks considering the fact that the chances of corrosion in reinforcement are reduced drastically.Although usage of Bio-concrete in comparison to the conventional Portland cement(is amicable but it isn't sustainable in the absolute sense. Design/formation of bacterial concrete is not

mentioned in IS codes or any other codes standardizing the process and the cost of this concrete is comparatively higher than conventional concrete i.e. about 7-28% more than conventional concrete(!!).The sprouting of bacteria is not suitable in any environment i.e it is a very time intensive and selective process.Talking about the investigations and research involved in calcite precipitation turn out to be capital intensive.Moreover, the bacteria that grow in concrete are not good for human health and the atmosphere and hence its usage should be limited to the structure

Table:Reaction conditions reported in the literature for production of CaCO3 via urea hydrolysis.

Purpose	**Urea(mM)**	**Ca2+(mM)**	**Urease Activity(mM/min)**	**Reference**
Sr90 sequestration	333	25	0.045	Fujita et al., 2000
Sr90 sequestration	330	0.025	0.042	Warren et al., 2001
Removal of Ca2+ from waste water	16	14	0.293	Hammes et al., 2003
Removal of Ca2+ from waste water	8	15	0.032	Hammes, 2002
Stone remediation	333	12-50	0.110	Stocks-Fischer et al., 1999
Stone remediation	333	340	0.02-0.12	De Muynck et al., 2011
Portland cement remediation	333	50	n/s	Ramachandran, 2001
Plugging of rock pores	333	0.25	n/s	Gollapudi et al., 1995
Biocementation	1500	1500	4-18	Whiffin, 2004
Carbonate precipitation	666	250	n/s	Okwadha and Li, 2010

1.4 Possible application of MICP:

1.4.1 *Removal of heavy metals*

At present there are a number of biological treatments (using microorganisms)which have been introduced to remove heavy metals from contaminated sites accumulated due to anthropogenic activities through *phytoremediation, bioaccumulation, biocoagulation, bioleaching, biosorbents and bioimmobilization* (Volesky 2001; Gadd 2000; Gazso 2001; Lloyd and Lovely 2001; Lin and Lin 2005; Achal et al. 2011) but these methods of treatment are expensive, time consuming and result in release of considerable amounts of immobilized or adsorbed heavy metals back to the environment (Achal et al. 2011).In MICP process, calcites can be incorporated heavy metals (e.g., Pb2+) onto their surfaces via substitution of suitable divalent cations (Ca2+) in the calcite lattice (Eq.6), after which these compounds are changed from soluble heavy metals to insoluble forms i.e., detoxify the heavy metals (Pan 2009; Achal et al. 2011). Li et al. (2013) reported that a few species of *Sporosarcina* and *B. lentus* urease producing bacteria were able to remove 88 to 99 % of heavy metals after 48 h of incubation

$$Pb^{2+} + HCO_3^- + OH^- \rightarrow PbCO_3 + H_2O \qquad (6)$$

1.4.2 *Removal of radionuclides*

MICP method stimulates ureolytic microorganisms to promote CaCO3 precipitation, which in turn leads to promote co-precipitation of radionuclides by substitution of Ca2+ ion and formation of radionuclide carbonate minerals.In living organisms, strontium is highly toxic and soluble; therefore, it can be readily passed through the food chain from contaminated soil or water. Additionally, strontium is capable of exerting long term health impacts due to its long half-life (28.8 years) (Singh et al. 2008). The mobility and carcinogenic effects of Sr affect groundwater usability (Lauchnor et al. 2013), and the conventional remediation techniques are expensive and ineffective (AbdEl-Sabour 2007). Strontium 90 exists in the environment as the Sr2+ ion, which has chemical similarity to Ca2+; therefore, Sr2+ can replace calcium ions in living system.Many researchers have successfully demonstrated the co-precipitation of 90Sr2+ into calcite by substituting Ca2+ in calcite crystal through MICP effectively (Fujita et al. 2004; Smith et al. 2004; Mitchell and Ferris 2005; Achal et al. 2012c; Brookshaw et al. 2012). Warren et al. (2001) found that 95 % of strontium was captured in the solid phase by MICP when *Sporosarcina pasteurii*was used.

1.4.3 *Bio-consolidation of soil and sand*

Bio-consolidation is involved in prevention or stabilization of erosion and increasing slope stability. Conventional techniques such as applying cement or chemicals are primarily used to improve soil; however these can lead to permanent soil and water contamination or air pollution. Additionally, these synthetic chemicals can be injected into the subsurface to bind sand grains together, increasing soil strength and stiffness. However, this method is expensive, difficult to distribute uniformly and introduces hazardous substances into the soil .The induction of CaCO3 precipitation binds sand grains together at the particle–particle contacts, which increases the strength and stiffness of the soil .The application of bioconsolidation can lead to a tenfold change in the primary properties of the sand such as permeability, stiffness, compressibility and shear

strength .Microbial grouting is far cheaper than chemical grouting cause of chemical reagents used and also the cost involved in the process.

1.4.4 *Bio-concrete or biocementation*

Biocement can improve soil shear strength through the production of soil particle-binding materials in response to the introduction of bacteria and cementation reagents into the soil (Ng et al. 2012). Different bacterial strains have been shown to produce various levels of urease activity ranging from 2.2 to 20 mM of hydrolyzed urea/min (Harkes et al. 2010; Stabnikov et al. 2013). Urease activity should not be too high or too low for successful biocementation because urease activity in the range of 4.4 to 9.5 mM hydrolyzed urea/min. increased the strength of biocemented soil.The cracks form in concrete due to aging and freeze thaw cycles; however, many researchers have reported the remediation of cracks by MICP of *B. pasteurii* and other *Bacillus* species (Ramachandran et al. 2001; Achal et al. 2013). Bioclogging of soil restricts water flow through soil and reduces its permeability. The permeability of soil was reduced significantly through accumulation of biomass and production of exopolymeric substances (Vandevivere and Baveye 1992; Ng et al. 2012). DeJong et al. (2010) reported a reduction of pore size, porosity, and permeability, as well as improvement of the stiffness and strength of the porous media matrix in response to MICP. Bernardi et al. (2014) recently reported the manufacture of bio-bricks by MICP and compared the effectiveness with that of conventional cement and lime treated bricks.

Figure: Biomanufactured bricks at Sharjah UAE (Image Source: GreenerBuildings Staff 2010)

1.4.5 CO2 sequestration

There is an urgent need to reduce the release of CO2 into the environment.In nature, CO2 is sequestered by chemical fixation of CO2 in the form of carbonate such as calcite, aragonite, magnesite and dolomite, but the reaction rate is very slow.Several studies confirmed that CO2 could be effectively sequestered into carbonate by CA from different organisms (Bond et al. 2001; Ramanan et al. 2009). Ramanan et al. (2009) investigated whether the addition of CA enzyme to reaction mixtures containing CaCl2 solution saturated with CO2, resulted in enhanced deposition of carbonate/bicarbonate salts. The geological sequestration of CO2 has also been accomplished by the injection of supercritical CO2 (SC-CO2) into deep geological environments, oil bearing formations, deep-seated coal beds and deep saline aquifers (White et al. 2003; Haszeldine et al. 2005; Mitchell et al. 2010). The critical over-saturation values necessary for

CaCO3 precipitation have been discussed by many researchers (Ferris et al. 2003; Dupraz et al. 2009). These environments are known to shelter extensive and active microbial communities that could possibly interact with the injected CO2(Amend and Teske 2005; Dupraz et al. 2009) (Fig. 7). Among many carbon capture and storage technologies, biotechnology using CA in an immobilized enzyme reactor at these plants holds great promise because it is viable and environmentally benign (Liu et al. 2005), and the generated carbonate minerals are safe methods of long term CO2 storage.

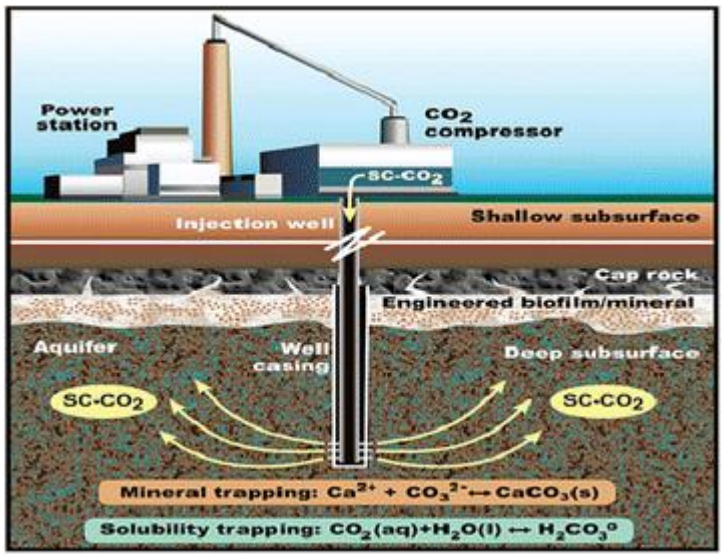


Figure:Schematic diagram of microbially enhanced carbon capture and storage (*Source* from Mitchell et al. 2010)

2.METHODOLOGY

There has been a lot of ongoing research on bio-cemented bricks and there a number of well revolutionary ideas that are being implemented on commercial scale like bioMASON(https://biomason.com/) which is a California based(United States of America) startup working on manufacturing bio-cemented brick using *Sporosarcina Pasteurii* without any usage of heat which is the most heat intensive part of brick manufacturing process(whereas as a traditional kiln requires a temperature of 2000°Cand releasing a huge amount of carbon dioxide in air).The founder was inspired by the coral reefs/structures that are known to exist in nature(oceans) and considering the fact that brick manufacturing accounts for 8% of the global carbon dioxide release.

2.1 **Problem Statement**

I was given to improve the strength/sturdiness of a sample of M-sand with any of the possible add-ons like PMMA(Poly(methyl methacrylate) ,also known as acrylic, acrylic glass, or plexiglass), Lunar soil,Epoxy resins etc but without incorporating any cement or any other commonly used binders .Few research scholars had been working on that before I arrived in Dr.Aloke's lab and they faced an issue is to make the specimen in the desired shape and finish without compromising the strength(compressive as well as sheer).They had been working on bio-consolidation but I was given to check the possibility of use of non-biological compounds such as epoxy(a binder) in combination with the normal bacterial inoculates and to test their compatibility with each other in controlled as well as uncontrolled conditions at various strengths. I had to come through with a whole new experimental setup which I was apprehensive at first and after having experienced a number of failed attempts until getting a noticeable/satisfactory result.

2.2 **Experimental Steps**

2.2.1

Initial hurdle was to decide the culture or the bacterial colonies to be used even though I was told to use ***Sporosarcina Pasteurii*** but I was curious to know why we can't use any other type as many other Urease positive bacterias are there like genera Sporolactobacillus, Sporosarcina, Bacillus, Clostridium and Desulfotomaculum which could also be used .

So there were certain criterion that have to be kept in mind while selecting the bacterial genera:

- *Size and shape of bacteria*
- *Soil particle size*
- *Nutrients required for incubation*
- *Type of chemical solution or reagent being used*

Table: Advantages and Disadvantages of direct addition of spores or micro-organisms in the bio-cement/bio-concrete

Methods	*Disadvantages*	*Advantages*	*References*
Addition of encapsulated microorganisms or its spore directly to the concrete	(1) Expensive method (2) complex procedure to prepare encapsulated microorganisms (3) cannot heal the crack that is propagated frequently at the same place	(1) High life time of microorganisms or their spores (2) Less effect on durability (3) strength and permeability (4) high biological concrete workability	A. Talaiekhozan, A.Keyvanfar, A.Shafaghat, R. Andalib, M.Z. Majid, M. A.Fulazzaky, M. Z. Rosli, C. T. Lee, M. W. Hussin, N. Hamzah, N. F. Marwar, H. I. Haidar,"A Review of Self-healing Concrete Research Development," Journal of Environmental Treatment Techniques, 2(1), 1- 11,2014.
Addition of attached microorganisms or their spores to the	(1) Decreasing of concrete strength (2) durability and permeability (3) Lesser	(1) Inexpensive (2) not complex (3) higher biological concrete workability (4) partially	A. Talaiekhozan, A.Keyvanfar, A.Shafaghat, R. Andalib, M.Z. Majid, M. A.Fulazzaky, M. Z. Rosli, C. T. Lee, M. W. Hussin, N. Hamzah, N. F. Marwar, H. I.

activated carbon or silica gel	protection for the microorganisms or their spores	can heal a crack that is frequently occurring at the same place	Haidar,"A Review of Self-healing Concrete Research Development," Journal of Environmental Treatment Techniques, 2(1), 1- 11,2014.
Circulation of microorganisms in the micro vessels throughout the concrete	(1) Very complex (2) Very expensive (3) Low biological concrete workability (4) No information about its effect on concrete strength.	(1) Able to repair a crack occurring at the same place (2) Highly durable (3) can heal a crack that is frequently occurring in the same place	A. Talaiekhozan, M. A.Fulazzaky,A.Keyvanfar, R.Andalib, M.Z.Majid, M. Ponraj, M. Z. Rosli, C. T. Lee, ShafaghatA., M. W. Hussin,"Identification of Gaps to Conduct a Study on Biological Self-healing Concrete," Journal of Environmental Treatment Techniques, 1(2), 62- 68, 2013
Addition of microorganisms or their spores into the hollow pipettes	(1) Complete information about its effects on concrete strength is not available (2) expensive (3) complex	(1) Higher lifetime of microorganisms or their spores (2) high biological concrete workability	A. Talaiekhozani, A. Keyvanfar, R. Andalib, M. Samadi, A. Shafaghat, H. Kamya, M. Z. Majid,M. Z.Rosli,M. A.Fulazzaky, C. T. Lee, M. W. Hussin,"Application of Proteus mirabilis and Proteus vulgaris mixture to design self-healing concrete," Desalination and Water Treatment. 52:3623–3630, 2014.
Addition of microorganisms or their spores directly to the concrete	(1) Presence of low microorganisms or reduces the lifetime of spores (2) cannot heal a crack that is propagated frequently at the same place	(1) Not effective on strength (2) not expensive (3) Noncomplex (4) high biological concrete workability	A. Talaiekhozani, A. Keyvanfar, R. Andalib, M. Samadi, A. Shafaghat, H. Kamya, M. Z. Majid,M. Z.Rosli,M. A.Fulazzaky, C. T. Lee, M. W. Hussin,"Application of Proteus mirabilis and Proteus vulgaris mixture to design self-healing concrete," Desalination and Water Treatment. 52:3623–3630, 2014.

2.2.2 Culutre preparation/protocol:

a. *Sporosarcina pasteurii* is a gram-positive bacterium able to survive in highly alkaline environments (pH~10) and is one of the bacterial species that can become a causative agent of MICP

b. *S. pasteurii* has the unique ability to secrete copious amounts of the enzyme urease. This enzyme acts as a catalyst, promoting an accelerated lysis of urea (a naturally occurring biochemical compound with widespread and abundant supply) in the presence of water molecules and has been known to not cause any dangerous diseases in human life forms.

c. *S. pasteurii* and its role in MICP are topics of active research and several issues relating to the mechanism of chemical precipitation are still not fully understood. In light of this, it is very important to have a set of consistent standardized protocols to accurately culture a suitably enriched stock of *S. pasteurii* to achieve MICP.

2.2.2.1 *Bacterial Culture*

Culture Bacteria - Agar Plate Medium Preparation

a. Assemble equipment and ingredients such as Petri dishes, flask, Tris-base, HCl, agar, Millipore water, pH-meter etc.Sterilize all containers by autoclaving at 121 °C before use.

b. Prepare 1 L of 0.13 M aqueous solution of Tris-buffer by mixing 15.75 g Tris-base with 1 L of Millipore water. To lower the pH level of the original solution (pH 10.4) add 2,800 µl of HCl (50% concentration). Check continuously using a pH-meter to set pH = 9.

c. Divide the 1 L buffer solution into two parts as follows:

i. Take 800 ml of this solution. Divide it equally into two parts of 400 ml each. Dissolve 8 g (NH4)2SO4 to one solution and 16 g yeast extract to the other solution.

ii. Take the remaining (200 ml of) solution and divide it again into two parts of 100 ml each. Mix 2 g (NH4)2SO4 to one. Add 4 g yeast extract and 4 g agar to the other.

d. Autoclave the 4 solutions separately after wrapping the respective flasks in Al foil and sticking autoclave tapes. NOTE: If a benchtop autoclave unit is used, the volume should be set to 500 ml (temperature and pressure automatically specified as a function of volume).

e. After taking them out from the autoclave, set the two 400 ml solutions aside for step 1.3.1 (below). Mix the two 100 ml solutions to have a 200 ml solution. Pour the mixture into 10 - 12 Petri dishes.

Culture Bacteria - Agar Plate Sample Preparation

a. Remove the bacterial stock from freezer (-80 °C) and allow it to thaw. After thawing properly, place the bacterial stock and the agar plate inside a biosafety hood.
b. Select the micropipette of smallest available dimension (0.5 - 10 µl is a good choice) to infest the tip with the *Sporosarcina pasteurii* stock. Streak an agar plate with the micropipette tip. Place the streaked agar plate inside a non-shaking incubator at 31 °C for 48 hr.
c. After 48 hr, remove the plate from the incubator and visually examine for the existence of single colonies. If there are no single colonies, then place it in the incubator for another 24 hr.
d. Repeat the process until single colonies are detected. Do not exceed 7 days of trial. NOTE: If single colonies do not appear even after a week, then it is concluded that the steps have not been followed properly and the entire process must be repeated from step 1.

Culture Bacteria - Final Sample Preparation

a. Mix the two 400 ml solutions (Tris buffer + (NH4)2SO4 and Tris buffer + yeast extract (1.5.1) together to obtain an 800 ml solution. Transfer 125 ml of this solution into a flask.
b. Perform a visual examination of the surface of the agar plate to identify regions with high concentration of single colonies. Gently nudge and break one of the colonies with a micropipette tip.

c. Dip the same micropipette tip into the 125 ml flask and stir it thoroughly to ensure that sufficient number of cells for robust multiplication get transferred. Place the flask in a shaking incubator at 150 rpm, 30 °C for 2 - 3 days. After 2 - 3 days, remove the flask from the incubator.

Culture Bacteria - Final Cell Count

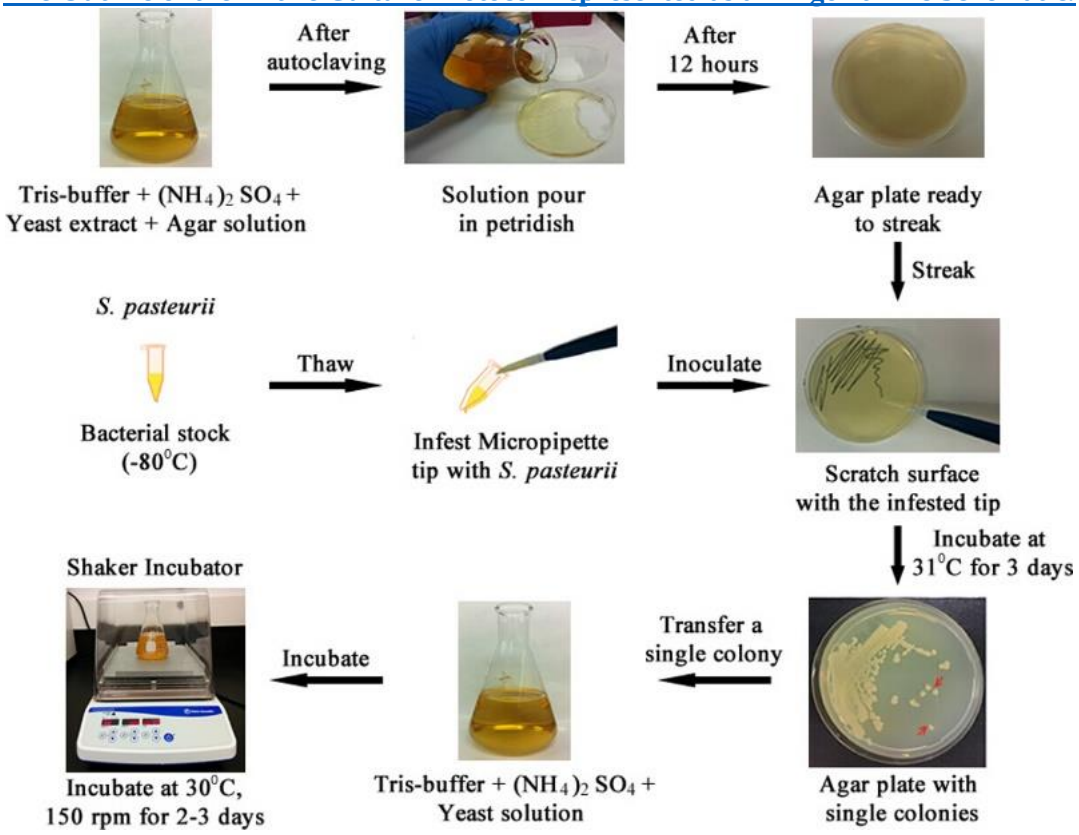
a. Perform serial dilution of the non-diluted culture solution using PBS to attain a dilution of at least ten million (10-7) to ensure countable single colonies appear. Draw seven parallel equidistant lines on one of the agar-plates.
b. Do this by drawing bold lines on the bottom surface of the Petri dish, prominent enough to be visible from top. Drop 3 little drops of non-diluted solution into one segment. Add 1 ml of non-diluted solution to 9 ml of Phosphate Buffered Saline (PBS) to obtain a 1:10 dilution.
c. Take a small aliquot (~ 0.1 ml) of this newly diluted solution with a pipette and drop 3 more small drops on the next segment. Transfer the newly diluted solution to a new flask and further diluted ten times (10x) by adding PBS. This brings down the dilution to 10-2 or 1:100.
d. Use this 1:100 solution in the next segment. Repeat this he process with small volumes of the freshly diluted solutions by successively transferring them to new flasks and continuously diluting ten-fold (10x) in tandem with PBS to obtain more and more dilute samples from 10-3 or 1:1,000 all the way down to 10-7 or 1:10 million into the last segment.
e. Perform Colony-Forming Unit (CFU) plate count to count the number of cells present in the agar plate after incubating the plate for 1 - 2 days at 31 °C. This gives a quantitative measure of the bacterial count in the undiluted sample. NOTE: The CFU value is measured based on the ability of the system to give rise to colonies under the specific conditions of nutrient medium, temperature and time assuming that every colony is separate and founded by a single viable microbial cell.
f. Seal the Petri dishes with self-sealing film and store remaining items in a refrigerator for future use.

2.2.3 Now to decide to make an external structure holding off the mixture of sand and any other additives that would support the structure enough until it'd b kept for bacterial treatment or for the culture to be added

Possible structures we took into consideration or one's that almost failed were-

1. Casting an aluminium die of the required specifications(ϕ20-25mm ; ℓ=50-60mm)
2. Using a large 150mL syringe as an outer periphery and using its plunger as an enforcer
3. Using small syringes with plastic straws cut and made into a circular arrangement.

The Outline of the Entire Culture Protocol Represented as an Algorithmic Schematic.



2.2.4 Decide what binder should be used of all the possibilities:

1. Using PMMA as a binder
2. Using epoxy resin
3. Using marble shavings

2.3 **Steps for preparing the M-sand:**

I. Collect m sand (I collected it from construction work going on near main building)

II. Visual inspection and cleaning of the sample

III. Keep in electric furnace at 100°C for around 3-4 hrs and check the moisture using rapid moisture meter method

IV. Take a sand sieve column to filter out the sand into different particle sizes

V. Now,the sieve column has a varied number of sieves namely more than 350µm,300µm,250µm,212µm,125µm and less than 125µm.

VI. Mix proportions of <125µm,125-212µm,212-250µm,250-300µm in ratio of 1:4:4:1 so as the total weight of mix is 1000gm or 1kg.

2.4 **Using Disposable polypropylene syringes (DispoVan) (***made sure to use an OHP sheet along the circumference of the syringe/cylinder so as to prevent the brick from being damaged)*

2.4.1 *Using M-sand and PMMA*

I. Take 30gm of the required M-sand mix and autoclave it to get rid of all the bacteria

II. Now taking various combinations of PMMA-15%,20%,25% by weight in the sand mixture.

III. Melt the weighted PMMA put in a glass beaker using a e;electric furnace at a temperature >170℃

IV. Make sure and take all the necessary precautions like wearing a face mask,gloves and glasses to cover your eyes.

V. Now pour the melted PMMA cautiously in to another beaker containing the sand mix and stir continuously till the mix shows a certain amount of homogeneity.

VI. Allow the mix to cool down for 20 minutes and then put it in the syringe which could be sealed at the other end using a glue gun and cotton/tissue papers.

VII. Let the specimen to cool down for atleast 1 hour at room temperature

VIII. At the end,,cure the specimen at temperature around 100℃ so as to enhance the solidification and giving the required porosity of the sample.

M-sand and PMMA treated sample

2.4.2 *Using M-sand and epoxy resin as a binder*

I. Take 30gm of the required M-sand mix and autoclave it to get rid of all the bacteria

II. Take various compositions of epoxy binder by weight(notably 15%,20%,25%,30%) so as to check till what strength we can possibly go to without the use of bacterial culture

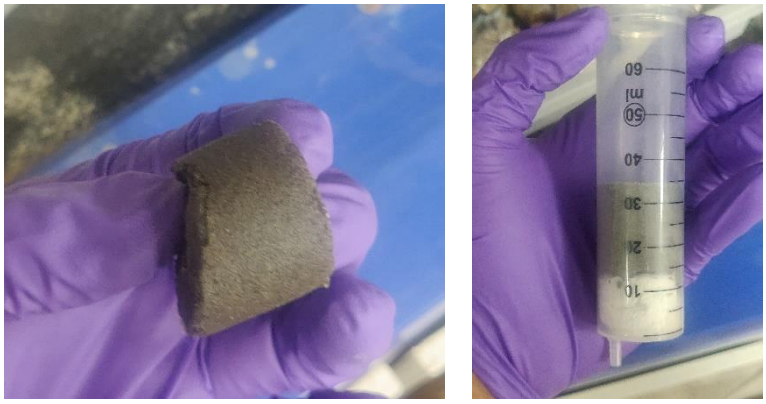
III. Take 100:147 w/w strength of hardener and resin of the required percent weight of the total mix using a **lab weighing scale**

IV. Mix the hardener and resin in a glass beaker and slowly pour the weighted sand and stir until mixture appears to show a certain amount of homogeneity.

V. Now pour this mix from beaker into syringe using your hands(it's must to wear gloves)

VI. Compact the sand using the plunger provided along the syringe and let it dry for atleast an hour

VII. Finally cure the specimen after taking it out from the syringe at a temperature of 100°C and let it cool down again at room temperature for 2 to 3 hours



M-sand and epoxy resin samples at varied concerntration

2.4.3 **Using 60 ml Disposable polypropylene syringes (DispoVan) and the bacterial culture**

1. Live S. pasteurii cells were collected by centrifugation and washed with saline water
2. The washing step was repeated until the pH of the supernantant became neutral (pH = 7) and then cells were resuspended in the culture medium
3. For CaCO3 precipitation experiments, S. pasteurii cultures were prepared in nutrient medium with urea and CaCl2 supplements
4. 0.1 g **glucose**, 0.1 g **peptone,** 0.5 g **NaCl**, 0.2 g **mono-potassium phosphate**, 2 g **urea**, 0.0012 g **phenol red dye** and 2 g **agar** to 100 ml distilled water. All chemicals were procured from Hi-Media, India.
5. All the liquid cultures were incubated in aerobic conditions at 30˚C with an orbital shaker operated at 120 rpm
6. Different experimental combinations were prepared to observe the role of bacteria and the effect of composition of growth medium on the initiation of CaCO3 precipitation in liquid culture
7. Sand samples were autoclaved at 121°C and 15 psi for 30 minutes.
8. However, the treatment was split into two series of treatment and added twice daily.
9. The MICP treatment was performed by introducing *10 mL of bacterial culture* and *10 mL of cementation solution* into the sand specimens at an interval of 12 h for a duration of 96 h.

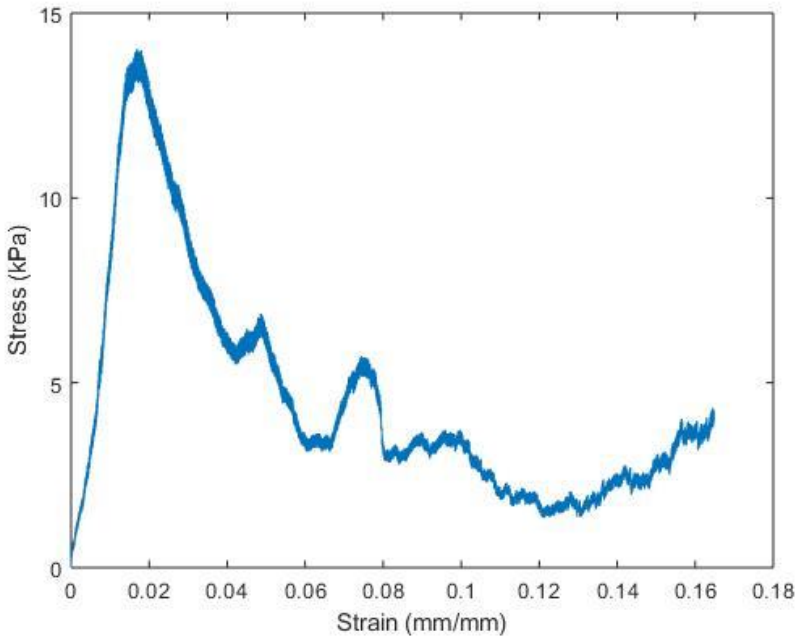
10. The treatments of the sand columns were performed inside a fume hood.
11. Upon completion of the treatments, all the sand columns were cured at room temperature for a duration of 14 days before the treated sand were being removed from their respective mould
12. Undergo mechanical strength testing (to determine strength of sample) and XRD + SEM (to determine the type of crystals).

Control (Sand + Media)

SP+ Synthetic Media + Urea+Calcium Lactate

3. RESULT

3.1 Compressive strength of the sample was determined using MicroUTM(universal testing machine) after machining/finishing the specimen to all surfaces to be flat and smooth.

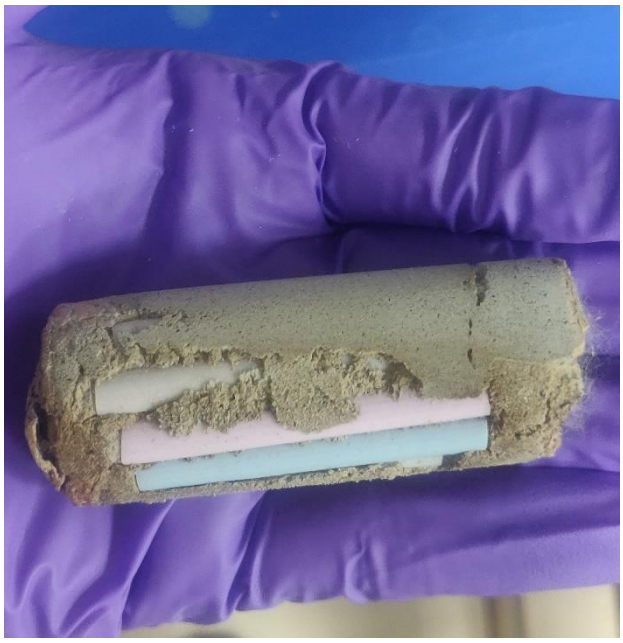


Stress v/s Strain curve for the MICP treated sample

3.2 Compressive strength of the bacteria-treated(MICP sample) specimen came out to be around **15kPa** which is lesser than a sample treated with both epoxy as well as the biomineralization which came out to be **18.3kPa**.

3.3 A comparison was drawn between PMMA and Epoxy treated sand specimen implying strength and sturdiness(aesthetic) of the epoxy specimen was better as compared former.

3.4 A failed attempt was made to incorporate straws(plastic daily use straws) as a supporting framework inside the syringe for the bacterial treatment was made, which came of as a surprise as the increased surface area at the interface/surface should have supported/catalyzed the process but on the contrary it inhibited the process



Sand containing straws axially inoculated with bacteria

3.5 Analysis of microbial precipitation was performed with the help of scanning electron microscopy (SEM) and X-ray powder diffraction (XRD) analysis to confirm the(precipitation of calcites in the sample (will be performed with the help Nitin Sir(PhD. Student)and Dr. Rashmi Dixit (Post Doc.) at Dr Aloke's lab)

3.6 Detrimental factors for MICP in the experiment

3.6.1 pH

pH plays a very big role in microbial system and the carbonate ions concentration at MICP process. MICP treatment utilizes organic acids [1] due to which there is an increase of both pH and concentration of dissolved inorganic carbon. Urease activity or urease hydrolysis by some bacteria has been extensively studied as compare to other biological systems for calcite production. This under surface activity of urease hydrolysing bacteria could produce NH^{4+} and bicarbonate ions causing an increased pH due to calcium carbonate production.

Urease catalyzes the hydrolysis of urea into CO_2 and ammonia leading into increased pH in bacterial environment. MICP treatment occurs at pH range of 8.3-9.0, as urease activity remains high at this pH [2]. Various workers studied series of events happening during ureolytic calcification using S. pasteurii [3].

3.6.2 Temperature

Urease catalyzed ureolysis is a temperature dependent process and suitable temperature range is from 20°C to 37°C because, ideal temperatures have a good effect on precipitation of calcite by bacteria and increasing the ability of the strain to form crystals. Other report states that increase in temperature will results in an increase in urease activity up to 60°C [4]. This feature is particularly interesting for those countries where temperature remains high. They concluded that if there is an increase of 10° over the range 5-35°C there will be an increase in urease activity also by the factor of 24. They also revealed that no urease activity was seen in soil temperature below 5°C, which could also be explained as at this temperature bacterial cells have limited activity. Most of the studies on temperature have been conducted at an ambient laboratory temperature of 20 ± 2°C. Calcium carbonate of calcite type can stay stable at room temperature [5].

3.6.3 Injection strategies

A suitable injection method is needed for successful MICP treatment. Microbial grouting can be achieved in several ways. The most important factor in order to achieve even calcite precipitation throughout the soil mass is uniform distribution of microbial cells followed by fixation inside the porous structure. Several ways of introducing/injecting bacterial cells in MICP treatment process have been studied. Prior mixing of bacterial cells and cement material leads to immediate flocculation of bacteria and crystal growth which may play an important role in treatment of surfaces [6]. This could lead to rapid clogging of injection point and surrounding areas pore space for many of the fine or medium sand. The two-phase injection is another strategy has been conducted, where the bacterial cell solution is injected first, followed by the cementation solution [7]. This strategy applied to prevent crystal accumulation around the injection point and led to a more homogeneous distribution of calcium carbonate. A more uniform distribution of calcite precipitation was achieved over a greater distance in the sand [8-10].

3.6.4 ***Effect of incubation period (h)***

The optimal incubation period was determined by incubating the ureolytic bacteria culture at different selected incubation periods ranging from 24 to 96 h with an interval of 24 h. The incubation period that promoted the highest enzyme activity was used for subsequent steps of the investigation.

3.6.5 Effect of urea concentration (%)

The influence of urea substrates with varied concentration for enzyme production was studied. Different urea concentration ranging from 2 to 10% (w/v) with an interval of 2% (w/v) was selected. The urea concentration that promoted the highest enzyme activity was used during bacterial cultivation for bio-cement experiment.

4.OPTIMIZATION POSSIBILITIES/FUTURE PROSPECTS

4.1 Study change of mechanical characteristics of healed cracked concrete due to bacterial calcite precipitation

4.2 MICP may not be completely environmental friendly, because ammonium and nitrate are formed during the ureolysis-driven process, which can be **toxic and hazardous** to human health and soil microorganisms at high concentrations .Look for a measure to mitigate this.

4.3 Improvement in finish quality of bio-cement.

4.4 The economic limitations to use of laboratory grade nutrient sources in field applications must be overcome(For example, corn steep liquor or lactose mother liquor may provide less expensive nutrient sources for successful commercialization)

4.5 Devise a method to implement MICP on large scale applications or commercial scale

4.6 Incorporate the use of polymers in the specimen(may increase compressive strength by a margin)

4.7 Crack remediation comparison between usage of bio-concrete and epoxy treatments

4.8 Study whether an Ureolytic Pathway could be successfully applied for Removing Heavy Metals from Wastewaters?

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