Invitro Study of Calcium Precipitation Induced by *Sporosarcina Pasteurii* Isolated From Urea Rich Soils

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Abstract
Sporosarcina pasteurii was isolated from urea-rich soils of agricultural areas in Peshawar. It was biochemically characterized as gram-positive bacilli, which are positive for catalase, methyl red and urease tests. The urease enzyme is an important contributing factor to biocementation because the enzyme alkalizes its vicinity, thus calcifying the calcium in the environment. The calcium precipitates were then manifested via electron microscopy, and it verified that the calcium precipitates were stable, polydispersed and cuboidal in morphology, thus confirming their calcite nature. The average sizes of calcites were estimated to be 50 nm in diameter, which positively assisted the self-healing/biocementation property of *S. pasteurii.*

Keywords: *Sporosarcina pasteurii,* Biocementation**,** Calcite, Calcium Precipitation**.**

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INTRODUCTION

Sporosarcina pasteurii (SP) is a non-pathogenic, gram-positive soil-dwelling bacillus, having an estimated size of about 4.0 μm in length and 0.5 μm in diameter[1]. The bacterium has gained global interest due to its renowned potency to precipitate calcite and solidify sand granules via microbiologically induced calcite precipitation (MICP) or simply biocementation[2]. The bacterium is characterized as alkaliphile, which can strive for higher pH in the range of 9 – 12. The paramount cellular property that assists the bacterial proliferation in an extreme alkali environment is its unique cell wall structure, particularly provision of increased peptidoglycan, teichoic acid, and amidation of free carboxyl groups[3]. Furthermore, *Sporosarcinapasteurii* possesses about 3.3 Mb genome, which is sequenced and investigated to own approximately 40% GC-rich regions thus alluding to its extreme-tolerant nature.The bacterium has seven identified genes for urease production, which is an accountable asset for biocementation[4]. The enzyme urease hydrolyzes urea to ammonia and carbonate ions, which in turn alkalizes the pH of the nearby environment, thus promoting speedy calcium precipitation. In addition, the negative charge on the cell surface

provides an excellent nucleation site, thus attracting the positive Ca^{++}, which positively encourages the MICP concept.

In comparison to other bacterial species, *S. pasteurii* was observed to have the highest negative charge on the cell surface, i.e., zeta potential -67 mV. In contrast, some slow mineralizing bacteria, such as *Bacillus* species, own less negative control, i.e., the zeta potential of -40.8 mV, respectively[5]. Aside from this, *S. pasteurii* is also characterized to function as halotolerant and alkaliphile organisms that proliferate its chances of survival in extreme conditions and provide desirable[6]. The research was prototyped as a mini-scale investigation of the phenomenon of calcium precipitation by *S. Pasteurii* invitro, by randomly isolating it from urea-rich soils.

METHODOLOGY

Sample Collection

Five soil samples were collected from random agricultural areas of Ring Road, Peshawar. The samples were stored in sterile zip lock bags, then transported to Microbiology Lab for further analysis.

Soil Analysis

The collected soil samples were then analyzed for pH, temperature, and presence of urea. The pH was recorded in 8 – 12 using pH strips. At the same time, urea was estimated by enzymatic hydrolysis of urea using urease powder[7], which increases the pH of soil solution by breaking down urea into ammonia and carbon dioxide. All the samples were then stored at room temperature.

Isolation Of *Sporosarcina Pasteurii*

For isolation of *S. pasteurii, a* sterile nutrient agar medium (Sigma Aldrich, Germany) was prepared in sterile Petri-plates. Further, serial dilution of soil samples in the concentration of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were prepared. A 1 mL sample was poured from each dilution on the solidified sterile Petri-plates. The plates were then undisturbed for 30 minutes in a laminar flow hood. After 30 minutes, the plates were incubated at 37oC for 24 hours in an incubator upright. Finally, all the Plates were morphologically analyzed.

Biochemical Characterization

The isolates were further sub-cultured and biochemically identified following Bergy's Manual Protocol.

Invitro Calcium Precipitation

The capability to precipitate calcium is assessed following the protocol documented[8]. For this purpose, sterile nutrient broth media is prepared by adding 1M 1M of urea and 0.75M of $CaCl_2.2H_2O$ to 100 ml broth media. Then it was cultured with the isolated *S. pasteurii* using a sterile inoculating loop. Finally, all cultured flasks are incubated in a shaking incubator at 37°C for 24 – 36 hours. After incubation periods, calcium precipitation in calcium deposits can be observed.

Characterization Of Precipitated Calcium

The calcium deposits are then morphologically characterized via scanning electron microscopy to assess their nanostructures. For this purpose, the thin smear of sample is loaded over a copper

grid which is then dried with the help of a mercuric lamp. The samples are then analyzed under variable magnifications.

RESULTS

Biochemical Characterization

S. pasteurii was biochemically characterized as gram-positive bacilli, having positive catalase, motility, methyl-red, urease and anaerobic tests. It was observed to be negative citrate, indole, and vogues-proskeur tests. Positive glucose fermentation and starch hydrolysis were also Observed The results are summarized in Table 1.

Table 1:*Biochemical Tests for S. pasteurii*

Biochemical Tests	Result for ***S. pasteurii***
Catalase	+
Motility	+
Methyl Red	+
Urease	+
Anaerobic	+
Citrate	_
Indole	_
vogues-proskeur	_
Glucose Fermentation	+
Starch Hydrolysis	+

Invitro Calcium Precipitation

S. pasteurii was observed for the property of calcium precipitation by alkalizing the media and precipitating calcium chloride supplemented in the media. It was manifested that urea is broken down to ammonia in the presence of the urease enzyme secreted by bacteria, which raises the pH of the medium from slightly acidic to alkaline. The alkaline pH promotes calcium precipitation, thus enhancing the process of biocementation. Results of which are shown in Fig. 1.

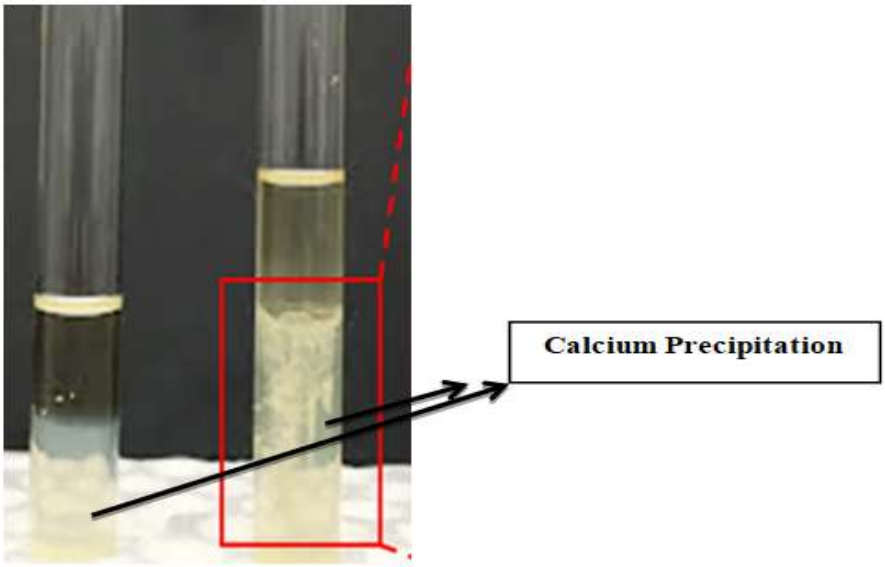

Fig. 1. *Induced Calcium Precipitation by S. pastuerii*

Characterization Of Precipitated Calcium

The precipitated calcium was morphologically tested by scanning and transmission electron microscopy. It was manifested that the precipitated calcium was calcite in morphology, having an estimated size range of about 50 nm. Calcite was observed to be stable cuboidal and rhombohedral in shape. Hence, it was manifested that *S. pasteurii* possesses biocementation property by forming stable calcite mineral, an essential mineral to be used as construction aggregates. Electron micrographs are shown in Fig. 2 and 3.

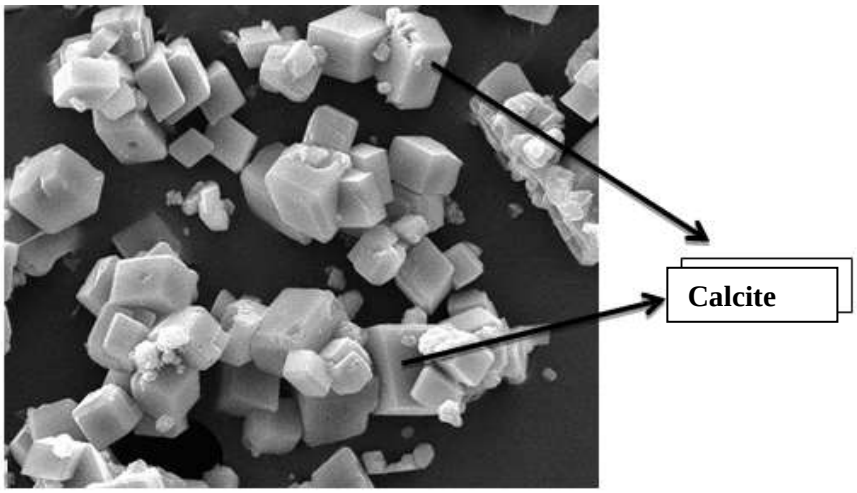

Fig. 2.*Scanning electron micrograph of calcite produced by induced calcium precipitation*

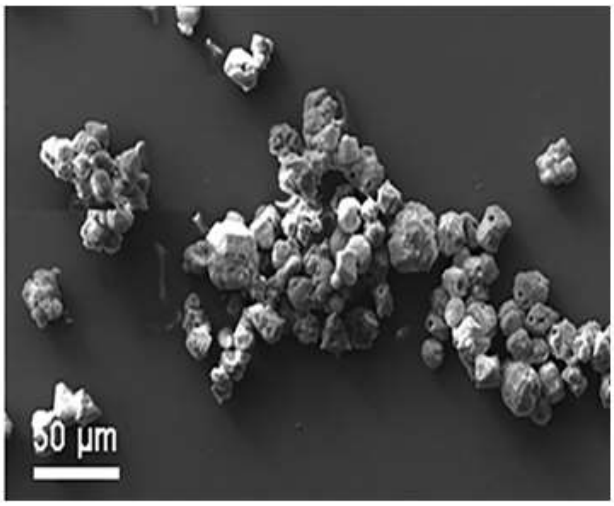

Fig. 3. *Transmission electron micrograph of calcite produced by induced calcium precipitation*

DISCUSSIONS

S. pasteurii possesses the capability to secrete urease enzyme, which calcifies the calcium salt in the media by alkalizing the media environment. This property was also manifested[9] which affirmed the calcium precipitates as vaterite and calcites. The calcium was observed to be actively precipitated on the cell surface of the bacterium, which alludes to its unique biocementation property, also verified the ureolytic property of *S. pasteurii*, which positively manifests its

microbially induced calcite precipitation. The calcite formation in the current study was estimated to be 50 nm, which is supported by the study, as nanosized calcites provide efficient biocementation capacity. Furthermore,[10] suggested that calcite precipitation properties can be engineered in various bacteria, which will, in turn, form a solid turning point in construction and material sciences to promote biomineralization and biocementation. Self-healing potentials of cracks in buildings can also upgrade this property.

CONCLUSION

From the research study, it was concluded that. *S,pasteurii* possess the potential to promote biocementation by secreting urease enzyme, thus alkalizing the nearby environment to aggregate calcium forming calcites. The construction industry functionally utilizes these calcites to create sturdy, self-healing buildings.

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