

Original Research Article

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A Study on Characterization of EPS and Media Optimization for Bacterial Calcium Carbonate Precipitation

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ABSTRACT

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Bacteria isolated from different habitats have been reported for their ability to precipitate calcium carbonate both in natural and laboratory conditions. Calcium carbonate precipitation was a known example of extracellular bacterial biomineralization and this process played an important role in calcium precipitation. In this study bacterium such as *Bacillus sp* and *Pseudomonas sp* precipitated calcium carbonate in an in vitro condition. Optimization of the medium was carried out and the extracellular polymeric substance that influenced the process of precipitation was extracted by both physical and chemical methods and its chemical analysis of carbohydrate and protein content was performed. The extracellular polymeric substance by these bacteria mediated calcium carbonate precipitation and thereby has new revolution in various applications.

Introduction

Biomineralization refers to the processes by which organisms' forms minerals. These minerals are composite materials comprised of both mineral and organic components. The organism uses cellular activities to direct the nucleation, growth, morphology and final location of the mineral that is deposited both intra and extracellularly (Weiner *et al.*, 2003). Number of studies has investigated the role of extracellular

polymeric substances on mineral precipitation and showed that it significantly alters the surface environment of bacterial cells, as well as providing an additional source of organic ligands and dissolved organic carbon. It alters the kinetic pathways of mineral nucleation and crystal growth. EPS is very common in natural environments, a major component of biofilms, makes it stable and protect it from harmful effects, as well as reducing water

loss from the cell to environment (Tourney *et al.*, 2009). EPS is composed of high molecular weight compounds, including polysaccharide, protein, nucleic acids, humic substances and ionisable functional groups like carboxylic, phosphoric, amino and hydroxyl groups. EPS has a large number of negatively-charged functional groups and form multiple complexes with many heavy metal ions and remove them.

Bacteria of the genus *Bacillus* are ubiquitous organisms found in the soil and are able to precipitate calcite on their cell constituents and in their micro environment. *Pseudomonas* sp also has ability to precipitate calcite crystals. Researchers have used standard nutrient media like nutrient agar medium or yeast extract medium to grow bacteria and precipitated calcium carbonate (V. Achal *et al.*, 2009). In this study, strains of both bacteria such as *Bacillus* sp and *Pseudomonas* sp which have precipitated calcium carbonate was selected for further investigation of media optimization with different carbon and nitrogen sources and extraction of EPS by both physical and chemical methods and characterize the composition of EPS and its influence in calcium carbonate precipitation. The applications of bacterially produced carbonate biominerals are improvement of the durability of buildings, remediation of environment (water and soil), sequestration of atmospheric CO₂, filler material in rubbers and plastics.

Materials and Methods

Screening of Calcium Carbonate Precipitating Isolates

Bacteria were isolated from soil of rhizosphere and biochemical identification tests were performed and identified. The obtained isolates were screened for calcium carbonate precipitation. Calcite, the stable

crystal modification of calcium carbonate, grows from hard water in clusters, which after a certain time form a solid scale firmly attached to the surface where the nucleation of crystals started. The presence of calcite was demonstrated on the special medium by inoculating into Triplicate B4 medium (calcium acetate 2.5g, Yeast extract 4g, Glucose 10g, Agar 18g/1000ml of distilled water, pH 7.2) incubated at 37°C for 28 days and the precipitates was purified by filtration, washed with sterile distilled water and dried at 60°C overnight (Chiara *et al.*, 2006).

Optimization of Calcium Carbonate Precipitation Medium

The medium that precipitates calcium carbonate was optimized by evaluating the effect of the precipitates with change in the carbon and nitrogen sources. The media components of the B4 medium were optimized by changing one parameter while remaining parameters to be kept unaltered. The carbon source was optimized by changing dextrose to glucose, sucrose and lactose. Similarly nitrogen source was optimized by changing yeast extract to beef extract and peptone. The isolates were inoculated and incubated at 37°C for 24 hrs, 48 hrs and 72 hrs and the growth of the organism that mediates calcium carbonate precipitation was monitored by obtaining the optical density (Jinlong Zhang *et al.*, 2014).

Extraction of EPS

The cell suspension of both the isolates was further subjected to EPS extraction by different methods. The physical method used for extraction of EPS was high speed centrifugation where the cell suspension was centrifuged at 20000 r/min at 4°C for 20 min. The supernatant was filtered. The chemical method used three extractants, EDTA2% at 4°C for 3hrs, formaldehyde at

4°C for 1 hr and formaldehyde plus NaOH (1N) at 4°C for 3hrs. After all the isolated treated with chemical agents was subjected to centrifugation at 20000 r/min at 4°C for 20 min and the supernatants were filtered. The filtrate was obtained by both the methods and was further used for characterization studies (Xiangliang Pan *et al.*, 2009).

Chemical Analysis of EPS

The chemical analysis of EPS obtained by both physical and chemical methods of extraction for both the isolates was assessed. The carbohydrate content of EPS was determined by the Phenol sulphuric acid assay with glucose solution as standard. (Dubois *et al.*, 1956). The protein content of EPS was determined by Lowry's method with bovine serum albumin as standard (Hong Liu *et al.*, 2002).

Results and Discussion

The isolates obtained after preliminary and biochemical identification tests were confirmed as *Bacillus sp* and *Pseudomonas sp* respectively. These bacteria were known for its ability to precipitate calcium carbonate in the B4 medium, which was confirmed by previous studies. The B4 medium was optimized by varying carbon and nitrogen sources. Among the carbon sources, glucose and lactose (Figure: 1& 3)

showed high growth rate than sucrose for both *Bacillus sp* and *Pseudomonas sp*. Among the nitrogen sources, beef extract and peptone (Figure: 2 & 4) showed high growth rate than yeast extract for both *Bacillus sp* and *Pseudomonas sp*.

EPS was extracted from both *Bacillus sp* and *Pseudomonas sp* by both physical and chemical method was further analysed for its carbohydrate and protein content. The maximum protein content was 9.5mg/g of *Bacillus sp* and 7.7mg/g of *Pseudomonas sp* by the chemical method extraction using EDTA (Figure: 5).The least protein content was obtained by the physical method of extraction. The maximum carbohydrate content was obtained by the chemical method of extraction using EDTA for both the organisms (Figure: 6).

The calcium precipitating activity of bacteria is the key factor of self-healing effectiveness of concrete crack. Marine bacteria with high calcium precipitating bacteria was screened and identified. The effects of influential factors such as carbon source, nitrogen source, spore inoculum and pH on the performances of bacterially induced calcium carbonate precipitation were investigated. This study laid a foundation for further application of bacterial self-healing of concrete crack (Jinlong Zhang *et al.*, 2014).

Figure.1 Optimization of Carbon Source – *Bacillus sp*

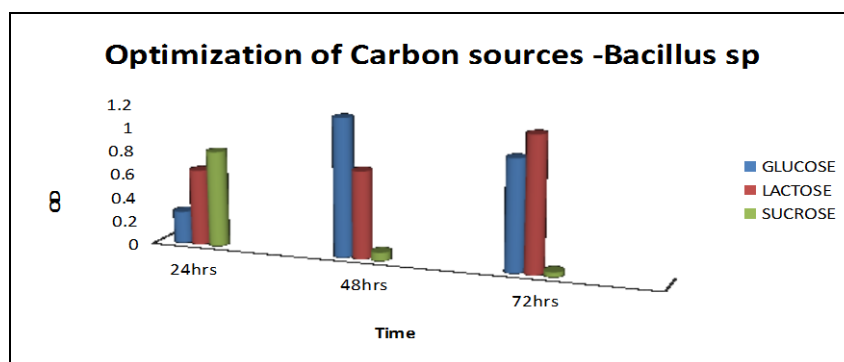


Figure.2 Optimization of Nitrogen Sources – *Bacillus Sp*

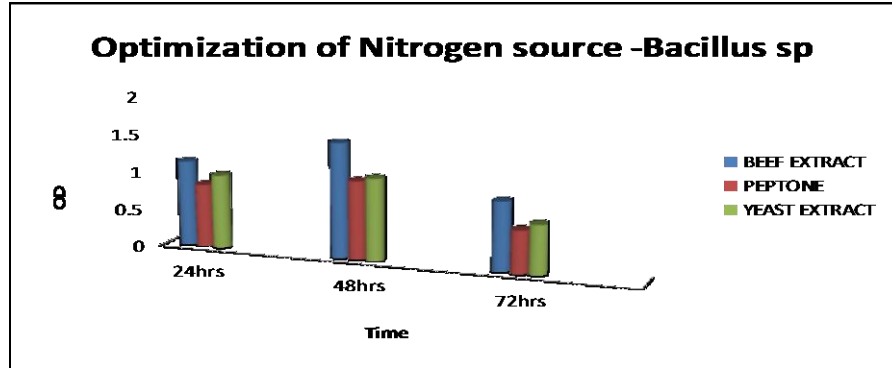


Figure.3 Optimization of Carbon Sources – *Pseudomonas Sp*

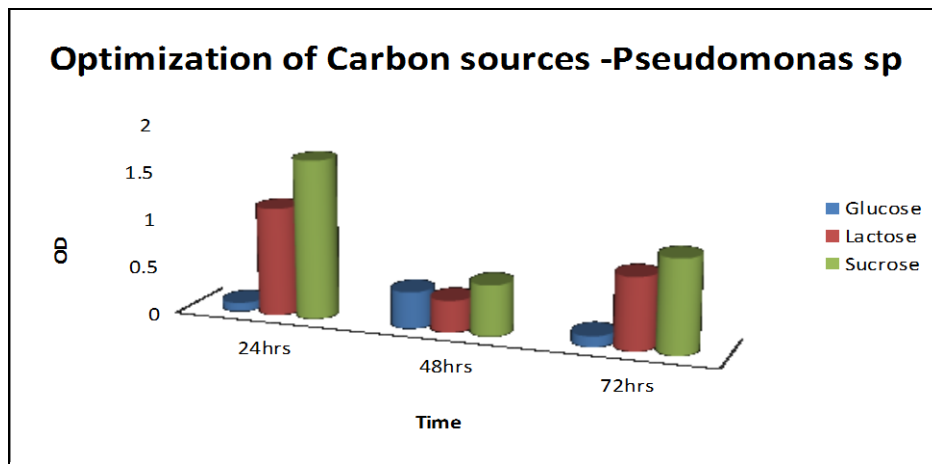


Figure.4 Optimization of Nitrogen Sources – *Pseudomonas Sp*

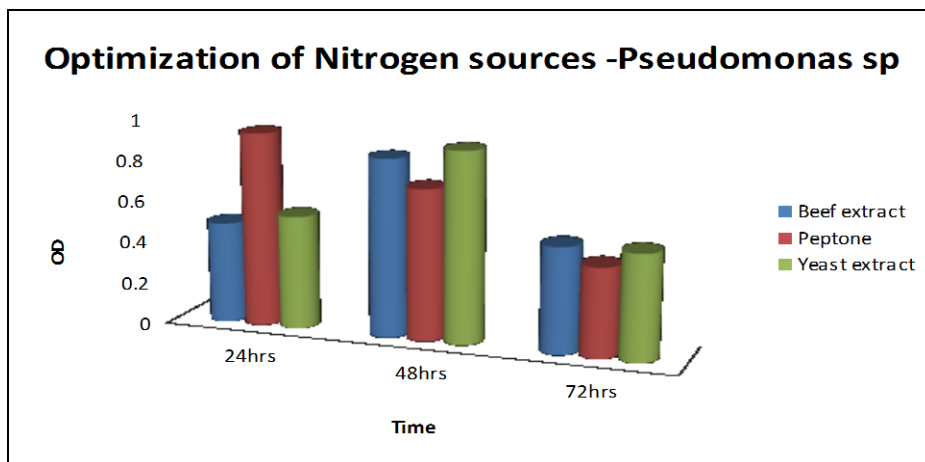


Figure.5 Chemical Analysis of EPS – Protein Content

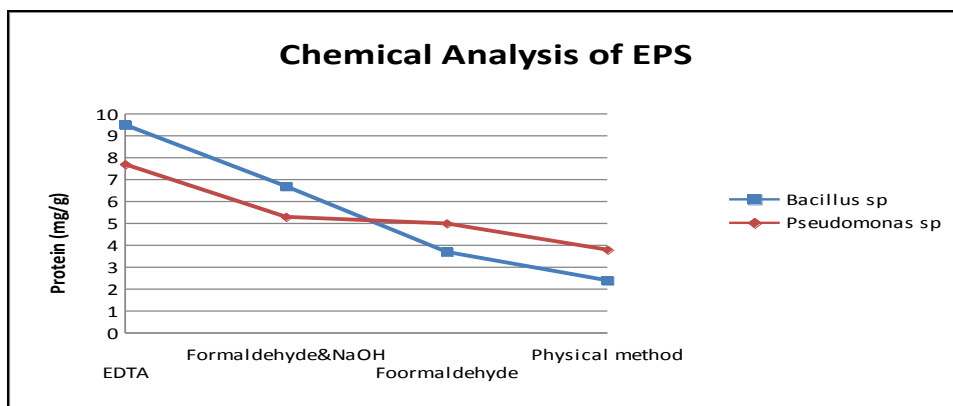
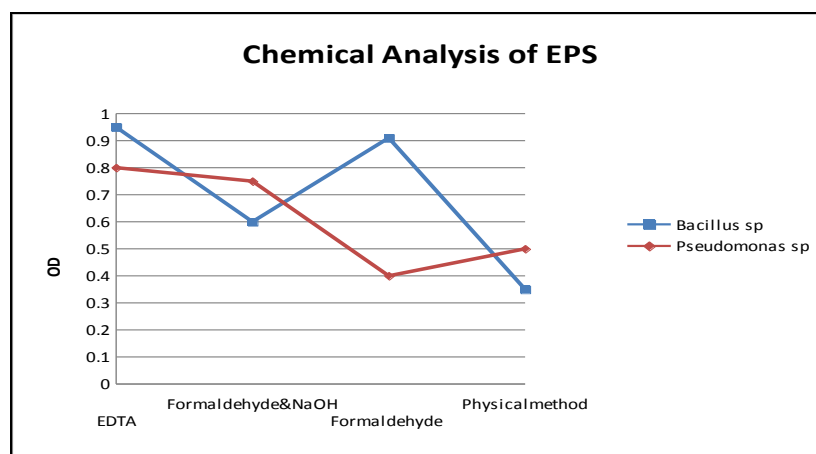


Figure.6 Chemical Analysis of EPS – Carbohydrate Content



Modified B4 showed a greater decrease in calcium ions than 295, for all strains tested, indicating that more calcium carbonate was produced in the former medium. Modified B4 gave greater calcium carbonate precipitation. In modified B4, *Pseudomonas putida* consumed on average 96% and *Bacillus subtilis* 28% calcium ion compared to the negative control without bacteria (Marcia *et al.*, 2011). The carbon and nitrogen sources used in the present study showed significant effect on the precipitation of calcium carbonate especially glucose and lactose among the carbon sources and beef extract and peptone among the nitrogen sources for both the organisms.

EPS had been extensively studied with regard to its physiochemical properties, interactions with contaminants and role in biofilm formation. The yield of EPS from biofilm varied greatly with the extraction methods. Compared with the physical methods, chemical methods significantly increased the extraction yield. The protein content of EPS obtained a yield of 1.70mg/g by EDTA method, 8mg/g by Formaldehyde method and 17.07mg/g by Formaldehyde-NaOH method (Xiangliang Pan *et al.*, 2010). The EPS extracted in the present study showed maximum yield of 9.5mg/g by *Bacillus sp* and 7.7mg/g by *Pseudomonas sp* by the chemical method, especially EDTA

extraction than the other chemical and physical method.

Biom mineralization usually involves metabolic activities of microorganisms and can occur in a biologically controlled, biologically influenced or biologically induced manner. Utilization of this natural process could be a turning point in the field of environmental engineering. Microbially induced biominerals by microorganisms are being extensively used in various fields ranging from removal of heavy metals and radio nucleotides to remediation of building materials. The present paper stands as significant demonstration on optimization and analysis of EPS by bacteria especially *Bacillus* sp and *Pseudomonas* sp that ultimately mediates calcium carbonate precipitation.

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