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# An Investigation of Biocement Production from Hard Water

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**Abstract:** Reduction of water hardness's samples by using urease positive bacteria to produce biocement was investigated in current research. In this research maximum urease activity of 5 bacterial strains in urea broth medium according to bacteria growth curve was studied. Urease activity of bacterial cells to precipitate carbonate was studied in 1.5M urea medium including serial dilution of $CaCl_2$ from 150 to 400ppm at 30°C for one week incubation. $CaCO_3$ yield was determined after centrifuging at 10016×g for 15min. Two natural hard water sources were used for biocement production. Scanning electron microscope and X-ray Diffraction methods were applied for morphological and elemental biocement analysis. The result showed that, high level urease activity was calculated 17.59Mm urease. $min^{-1}.OD^{-1}$ at the end of logarithmic phase of *Proteus vulgaris* PTCC 1079 growth curve as a dominated strain to produce urease enzyme. Minimum and maximum $CaCO_3$ in media including different serial dilution of $CaCl_2$ was determined 100 and 1030 $mgL^{-1}$, respectively for this bacterium. Optimum growth and urease enzyme production were obtained at 3 and 2M, respectively. Calcium was searched as the main element with same score (3000) and structure in biocement and ordinary cement by SEM and XRD analysis.

**Key words:** Biocement · Urease · *Proteus vulgaris* · Hard water

## INTRODUCTION

The level of water hardness is related to the amount of dissolved minerals such as calcium and magnesium in the water. Common calcium-containing minerals are calcite ($CaCO_3$) with highly variable forms and colors [1]. Waters can be divided from very soft to very hard with calcium concentration from 0-70 up to 530 ppm, respectively. At low hardness levels, water could become acidic and corrosive. There is a weak inverse relationship between water hardness and cardiovascular disease in men or eczema in children [2-10]. The World Health Organization has reviewed the evidence and recommended the maximum and minimum levels of calcium (40-80 ppm) and magnesium (20-30 ppm) in drinking water and a total hardness expressed as the sum of the calcium and magnesium concentrations of 2-4 mM/L [5, 7].

The Hard water can cause "scale" (mineral build up in pipes and plumbing fixtures) and reduce the effectiveness of soap for bathing and laundry. Most water softeners operate by ion exchange which trades (exchanges) sodium ions for calcium and magnesium ions [11].

The precipitation of calcium carbonate ($CaCO_3$) can mediate by microorganisms and producing both massive limestone deposits and small crystal forms [12].

These deposits of calcium carbonate which formed mediate by microorganisms, called Biocement or Microbial initiated Carbonate Precipitation (MICP). It is a technology that uses microorganisms to initiate crystallisation of carbonates to form a high strength cemented product by enzymatic pathway, especially urease. Urease activity is widespread amongst bacteria and it has been the approach used most often for applied MICP for production of calcite [13-15]. This enzymatic reaction can generate carbonate ions without an associated protons production. When this hydrolysis occurs in a calcium-rich environment, calcite (calcium carbonate) precipitates from solution forming a solid-crystalline material) [16].

Three main groups of organisms including photosynthetic organisms, sulfate reducing bacteria and nitrifying bacteria can induce MICP through their metabolic processes via remove $CO_2$ and increase the pH, dissimilatory reduction of sulfate and involved in the nitrogen cycle (ammonification of amino acids and urea hydrolysis) [17-19].

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Biocement has many advantages such as the process essentially replicates the formation of sandstone on a much shorter timescale, it is particularly suited to in-situ application, the reagents for biocement are produced at relatively low temperatures compared to ordinary cement where production involves heating ingredients to temperatures of up to 1500°C, it has the potential to be used as an eco-construction material as it has the potential to require less energy to produce the cement and hence involves the release of less greenhouse gases than production of other brick/cement construction material, it has been shown to achieve mechanical strengths that are comparable to conventional cement and multiple treatments can control the strength developed, the porosity and look (texture, color) of the original particulate matter is largely maintained, ie the use of sand in the biocement process would result in a sand colored and textured block, similar looking to sandstone and effects can be produced using multiple sand colors, the cementation process can be conducted *in-situ* without disrupting the structure of the original soil, biocement has the potential to be used in both unsaturated and saturated (including marine) applications [20].

The objective of this research was to develop suitable bacterial strains with maximum urease activity to act a catalyst in calcium-rich water samples (hard water) as a natural sources to reduce the water hardness and produce biocement as a valuable material.

## MATERIALS AND METHODS

**Samples and Culture Media:** The bacteria strains used in this research were *Staphylococcus aureus* PTCC 1112, *Staphylococcus epidermidis* PTCC 1435, *Staphylococcus saprophyticus* PTCC 1440, *Klebsiella pneumoniae* PTCC 1053 and *Proteus vulgaris* PTCC 1079 which were originally obtained from Persian Type Culture Collection, PTCC, Tehran-Iran. Culture was plated on Nutrient agar medium (Merck) and classic physiological characteristics were tested according to Bergey's Manual of Determinative Bacteriology [21].

Two samples of hard water sources were collected from tap water of Fashk village in the Tafresh city, part Farah, in 270 km southwest of Tehran and Kobar dam in Qom city, in 170 km southwest of Tehran and transferred to the laboratory for further tests.

**Urea Hydrolysis Test:** The colonies of strains were cultured on Urea agar medium (Merck) [2% urea and 10% phenol red indicator] and incubated at 37°C for 24 hours. A change in color of the media culture from yellow to pink indicates the presence of urease enzym in the sample. The diameter of colorful zones was measured in mm on agar medium and the largest halo was recorded for further experiments [22].

**Specific Urease Activity Analytical Method:** Specific urease activity was defined per unit biomass spectrophotometrically at 600 nm and was calculated according to following equation:

$$\text{Specific urease activity } (mM\,urease.\ min^{-1}.OD^{-1}) = \frac{\text{Urease activity } (mM\,urease\ hydrolysed.\ min^{-1})}{\text{Biomass } (OD_{600}\ nm)}$$

Ammonium concentration was determined by a modified Nessler method as described by Greenburg *et al.* [23]. Standards were prepared from analytical grade $NH_4Cl$.

**Bacterial Inoculums Culture:** Inoculums' culture of the best urea hydrolyser sample was performed in 100 ml Erlenmeyer flasks containing 100 ml of sterile nutrient broth medium and incubated at 37 °C for 24 hours. Bacterial cell density was adjusted on 0.8-1 at 600 nm by UV-VIS scanning spectrophotometer, UV 2101 pc, Shimadzu [22].

**Bacteria Growth Curve and Urea Hydrolysis Phases:** Growth pattern curve of the best urea hydrolyser sample was inoculated with 3% of inoculums culture in urea broth medium (Merck) and incubated at 37°C for 60 hours. The growth was investigated with determining cell cultures density at 600 nm every 2 hours using UV-VIS scanning spectrophotometer, UV 2101 pc, Shimadzu and compared with blank (culture in Nutrient broth medium). Urea hydrolysis was determined by detection of ammonium concentration. Sample was immediately centrifuged at 10016 ×g for 10 min to remove bacterial cell and the absorbance was measured at 425 nm. During these periods pH variation were determined by pH meter, Metrohem 827 [23].

**Urea-tolerance Levels:** The best urea hydrolyser sample inoculum (3%) was added to 1, 1.5, 2, 2.5, 3, 3.5 and 4M urea concentrations and incubated at 37°C. The visual turbidity was determined by cell cultures density at 600 nm [23]

**Biocement Production *In-vitro* Test:** The batch cultures medium were prepared by supplementing the best urea-sub tolerance level medium broth with $CaCl_2$ salt

concentrations of 150, 200, 250, 300, 350 and 400 mg/ml. 50% of inoculums' culture of the best urea hydrolyser sample (OD600 nm, 0.8-1) was added to each concentration and incubated for one week at 37°C. The pellet of calcite was collected by centrifuging at 10016 ×g for 10 min and dried at 50°C. The total pellet content was measured according to bacterial dried mass cell [16].

**Determination Water Hardness by EDTA Titration:** In this procedure the total water hardness was determined by measuring the concentrations of calcium and magnesium ions in water samples by titration. The sample is first adjusted to a pH of 10 using a sodium hydroxide buffer solution, a few drops of Eriochrome Blak T (0.5% wt/vol) indicator was added and sample was titrated with 0.01M sodium ethylene, diamine tetra-acetate (EDTA) solution to obtain a reaction from any calcium and magnesium in the water. EDTA draws the calcium and magnesium ions into a complex, so neither one has free ions in solution. The indicator initially turns red in the presence of calcium and magnesium then turns blue when enough EDTA solution has been added to combine with all calcium and magnesium ions. The total hardness of the sample is calculated using the precise volume of EDTA solution added when the indicator changes color, as well as the EDTA concentration, in mol/L. The $CaCO_3$ concentration was calculated according to following equation:

$$CaCO_3\ (mgL^{-1}) = [\text{vol. EDTA (ml)} \div \text{sample volume (L)}] \times [CaCO_3\ (1mol) \div EDTA(1mol)]$$

To determine the individual calcium hardness Murexide indicator was used. The pH of water sample was adjusted to 12-13 by using sodium hydroxide solution, a few drops of Murexide indicator (2.4% wt/vol) indicator was added and sample was titrated with 0.01M EDTA solution to the endpoint, which marked by a colour change from pink to purple [24]

**Biocement Production from Natural Hard Water Sources:** 100 ml of natural hard water samples were filtered by 0.45 μm Milipore filter. 1.5M filtered urea was added. 50% of inoculums' culture of the best urea hydrolyser sample (OD600 nm, 0.8-1) was added to each concentration and incubated for one week at 37°C. The pellet of calcite was collected by centrifuging at 10016 ×g for 10 min and dried at 50°C. The total pellet content was measured according to bacterial dried mass cell [16].

**Comparison of Biocement and Common Cement Structures by Scanning Electron Microscope and X-ray Diffraction Methods:** The final dried biocement was prepared to morphological analysis using a LEO 440i scanning electron microscope (SEM) equipped with Energy Dispersive Spectroscopy (EDS) and X-ray Diffraction (XRD). Wave-length dispersive spectroscopy was used to detect elements. Common cement was used as a blank sample.

## RESULTS AND DISCUSSION

Maximum urease enzyme production was determined by *Proteus vulgaris* PTCC 1079 in the end of log phase of bacterium growth curve (Table 1 and Fig. 1). This bacterium showed the maximum pH shift from 7 to 10 Cement industry is one of the most important environmental pollutant sources. Emissions of airborne pollution in the form of dust, gases, noise and vibration when operating machinery and during blasting in quarries are the major problems in this industry. It is the second largest $CO_2$ emitting industry behind power generation which produces about 5% of global man-made $CO_2$ emissions, of which 50% is from the chemical process and 40% from burning fuel [25]. The amount of $CO_2$ emitted by the cement industry is nearly 900 kg of $CO_2$ for every 1000 kg of cement produced [26]. So uses of green technology such as biocement production could be important to reduce environmental pollutions.

Maximum potential urease capacity was determined 17.59mM urease. $min^{-1}.OD^{-1}$ by *Proteus vulgaris* PTCC 1079 (Fig. 2). According to the results, this bacterium was collected for further analysis.

This microorganism was sufficient for biocementation process without additional processing such as concentration and cell lysis.

Urea is a major nitrogen resource in aquatic and soil ecosystems which a diverse section of the biota such as many bacteria, several species of yeast and a number of higher plants has evolved with the ability to hydrolyse it, through the action of urease enzyme [16, 27]. *Pseudomonasaeruginosa, Alcaligenes eutrophus, Bacillus megaterium, Klebsiella aerogenes, Sporosarcina pasteurii, Proteus vulgaris, Proteus mirabilis, Helicobacter pylori* and *Ureplasmas urealyticum* are the most popular bacterial strains which known as urease producer [29].

Table 1: Comparison of urease activity test based on color zone on urea agar medium and pH varition

| Bacterial strains | *Staphylococcus aureus PTCC 1112* | *Staphylococcus epidermidis PTCC 1435* | *saprophyticus Staphylococcus PTCC 1440* | *Klebsiella pneumoniae PTCC 1053* | *Proteus vulgaris PTCC 1079* |
|---|---|---|---|---|---|
| Urease activity zone (mm) | 6 | 4 | 5 | 2 | >10 |
| Maximum pH variation | 8 | 7.5 | 8 | 7.5 | 10 |

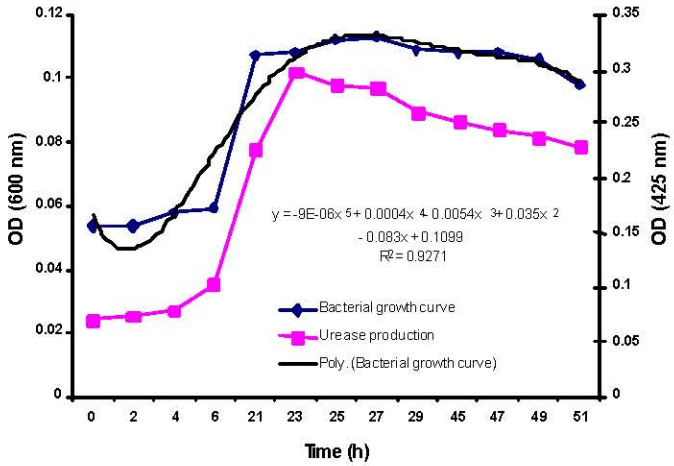



Fig. 1: Growth pattern curve and urease enzyme production by *Proteus vulgaris* PTCC 1079.

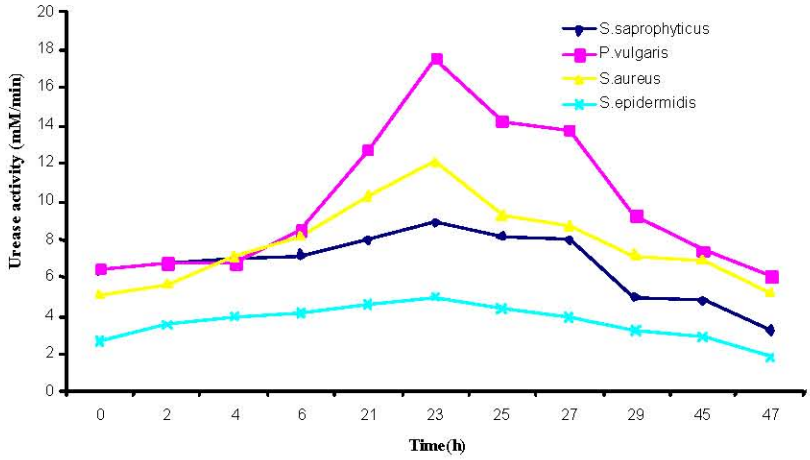



Fig. 2: Specific urease activity of *P.vulgaris*, *S. saprophyticus*, *S. aureus* and *S.epidermidis*. Cultivation was conducted at 37°C.

Whiffin [16] showed that *Proteus vulgaris* produced less levels of urease activity (10 mM urease. $min^{-1}.OD^{-1}$) compared to strain PTCC 1079 that applied in the present research.

The genera *Proteus* is one of the famous urease positive bacteria that have been reported with substrate induction [13,16,30]. The urease of this class can be induced in presence of urea. In these bacteria, the concentration of ammonium plays no role in the regulation of urease. Instead, urease can be induced to activities that 5 to 25-fold higher in presence this substrate [16,29,31].

McGenity and Sellwood [12] showed that Bacteria can mediate the precipitation of calcium carbonate ($CaCO_3$), producing both massive limestone deposits and small crystal forms. There are two phases for crystals formation, firstly the formation of nuclei (nucleation) whereas new crystals are formed and secondly a phase whereas very few new nuclei are formed and the growth of existing crystals dominate [32,33]. Nucleation is a complex phenomenon which is affected by temperature, the degree of over-saturation, the presence of other surfaces (e.g. dust, vessel walls and colloids) and bacterial cells [34]. It has been suggested that bacterial

Table 2: Concentration of $CaCO_3$ production in media including serial dilution of $CaCl_2$ from 150ppm to 400ppm by *Proteus vulgaris* PTCC 1079

| *Serial dilution of $CaCl_2$ (ppm)* | *150* | *200* | *250* | *300* | *350* | *400* |
|---|---|---|---|---|---|---|
| $CaCO_3$ production ($mgL^{-1}$) | 58 | 67.5 | 78.7 | 81.7 | 988.7 | 991.7 |

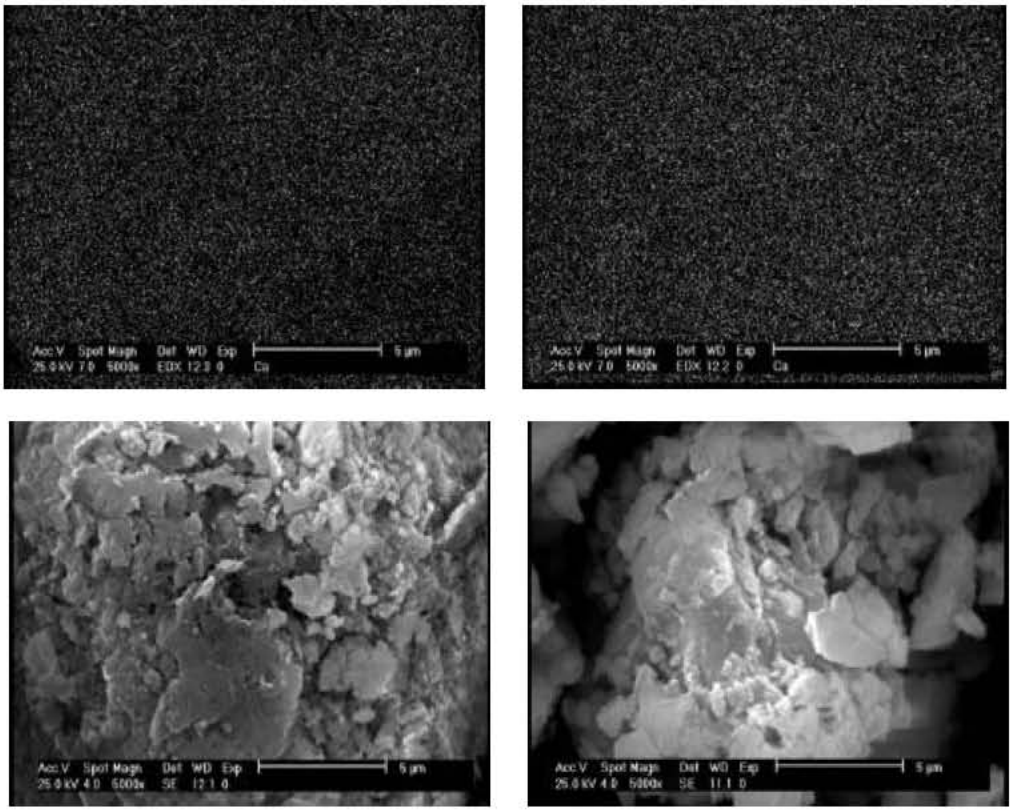


Fig. 3: Comparison of Biocement (left) and ordinary cement (right) structure by scanning electronic microscopy equipped with EDS (20keV). The Au is related to coating the samples with gold.

cells themselves can act as nucleation sites for the formation of crystals [35].

Minimum and maximum $CaCO_3$ in media including serial dilution of $CaCl_2$ from 150 to 400ppm was determined as 100 and 1030 $mgL^{-1}$, respectively (Table 2).

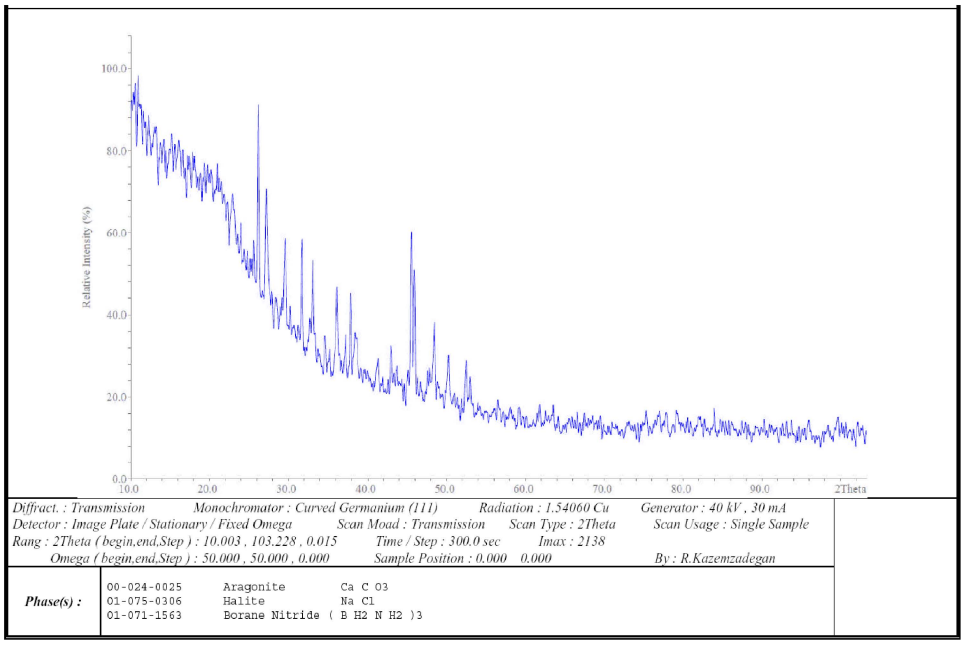
The result showed that whole cell of *Proteus vulgaris* PTCC 1079 was tolerant to concentrations of up to 4 M urea. But optimum growth and urease enzyme production were obtained at 3 and 2M, respectively.

This high level of tolerance for cementation conditions further confirmed that this organism was suitable for biocementation. Alkaliphile pH is one the major parameter to precipitate cement in natural sources. In this condition microorganism could be cultivated in non-sterile conditions with a minimum of upstream and downstream processing [16].

Water hardness by EDTA titrations for two natural sources (Fashk village and Kobar dam) were determined 290 and 640 $mgL^{-1}$ respectively.

Scanning electron microscopy-energy dispersive spectroscopy (SEM-EDS) and XRD detected calcium as a main element, in biocement and ordinary cement, were dispersed homogeneously (Figs. 3 and 4). The SEM images showed that the precipitated crystals bigger than 100 $\mu$m were formed clusters. In the both cements calcium score were the same with 3000 but in biocement carbon source also was search with 72 score. In XRD analysis the calcium score was searched with 66 and 59 in biocement and ordinary cement respectively (Fig.4).

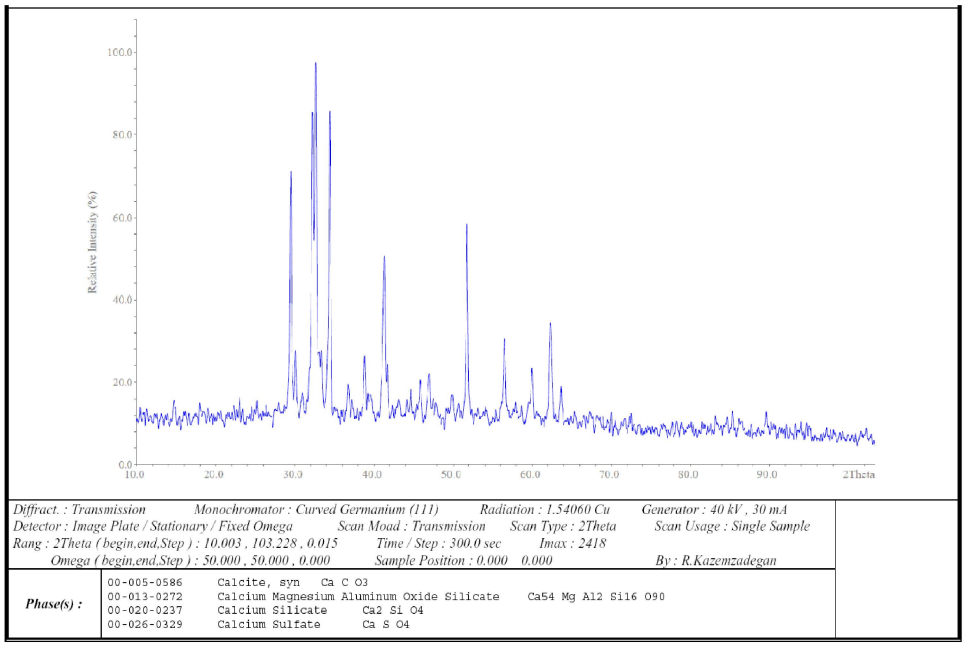
Studies on morphological characterization through the technique of scanning electronic microscopy equipped with EDS (20keV), for biocement and ordinary cement as a blank sample, showed a morphology, which consisted of agglomerates of thin particles magnitude ($d < 100$ $\mu$m) as shown in Figure 3. The main element was searched as calcium with the same score 3000 in both cements. Because of bacterial medium which used




(a)

**Pattern List**

| Ref.Code | Score | Compound Name | Chem. Formula | Matched Lines | Total Lines |
|---|---|---|---|---|---|
| 00-024-0025 | 66 | Aragonite | Ca C O3 | 44 | 54 |
| 01-075-0306 | 47 | Halite | Na Cl | 8 | 9 |
| 01-071-1563 | 62 | Borane Nitride | ( B H2 N H2 )3 | 124 | 159 |




(b)

**Pattern List**

| Ref.Code | Score | Compound Name | Chem. Formula |
|---|---|---|---|
| 00-005-0586 | 27 | Calcite, syn | Ca C O3 |
| 00-013-0272 | 59 | Calcium Magnesium Aluminum Oxide Silicate | Ca54 Mg Al2 Si16 O90 |
| 34 | 34 | | |
| 00-020-0237 | 25 | Calcium Silicate | Ca2 Si O4 |
| 00-026-0329 | 34 | Calcium Sulfate | Ca S O4 |

Fig. 4: Comparison of Biocement (a) and ordinary cement (b) structure by XRD analysis.

to produce biocement, carbon source also was searched only in biocement.

X ray diffractometry was used to examine the biocement and ordinary cement as a blank sample. Results from the diffractometry revealed in their spectra (Figure 4) the presence of Aragonite, Borane Nitride and Halite with score 66, 62, 74 and Calcium Magnesium Aluminium Oxide Silicate, Calcium sulfate, Calcite, Calcium Silicate with score 59, 34, 27, 25 in biocement and ordinary cement respectively.

The mechanical tests put emphasis on the values of mechanical properties for biocement compositions are necessary and must be done in next research.

In conclusion, *Proteus vulgaris* PTCC 1079 as a positive urease bacterium is suitable for use under biocementation condition in hard water. It is functional in room temperature and can tolerate concentrations of up to 400 ppm calcium.

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