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CALCIUM PRECIPITATING BACTERIA ON BIOMIMITIC COATING MATERIAL

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Abstract: The possible mechanism on calcium crystals formation by marine thermophilic bacteria has been explained. So many factors involved for crystal formation such as pH, temperature, salt etc. In the present study microbiological aspects of crystal formation. The natural bacterial growth curve and pH measurement were done. The Marine thermophilic calcium precipitating bacteria are rod shaped bacteria observed by Epi-fluorescence microscope and paint coated mild steel coupons with calcium precipitating bacteria harvested from B4 medium. The SEM studied for the calcium precipitating bacteria was around 1µm cell. FTIR and XRD were analyzed and indicated the presence of calcium carbonate with the bacterial pellets. It indicates that the presence of calcium coat in the outer surface of bacteria found.

Keywords: Calcium precipitating bacteria, Thermophiles, FTIR and XRD

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INTRODUCTION

Calcium carbonate (CaCo3) is the one of the most common minerals on earth. Its precipitation is a common phenomenon, forms natural rocks and exists in environments such as marine water, fresh water, and soils (Ehrlic, 1998; Castainer *et.al* 1999). Its forms oolitic, fossiliferous and considerable limestone in the sediments. The increase in the concentration or decrease in the solubility of the calcium or carbonate in solution causes the natural precipitation of CaCo3.

Calcium carbonate precipitation is a general process in the bacterial world under appropriate conditions (Bang *et al* 2001). Some bacteria and fungi can induce precipitation of calcium carbonate extracellularly through a number of process that include photosynthesis, ammonification, denitrifycation, sulfate reduction and anaerobic sulphide oxidation (Castainer *et al* 2000) can induce precipitation of calcium carbonate.

Knorre and krumbein (2000) concluded that microbilogical carbonate precipitation (MCP) occurs as a byproduct of common microbial processes, such as photosynthesis, urea hydrolysis, and sulphate reduction. These metabolic process increase the alkalinity (increase pH and dissolved inorganic carbon content) of the environment and thereby favour CaCO3 precipitation. The negatively charged nature and specific functional groups of microbial cell walls favour the binding of divalent cations (Ca^{2+} and Mg^{2+}), thereby making microorganism's ideal crystal nucleation sites. Microbial extracellur polymeric substances are also an important factor in precipitation, either through trapping and concentration of calcium ions or as a result of specific proteins that influence precipitation. Kawaguchi and Decho (2002) suggested that specific proteins present in biological extracullar polymeric substances cause the formation of different CaCO3 polymorphs.

A new approach to obtain, on monuments, the precipitation of new calcite inside the stone porosity has been proposed with application of living culture of selected calcinogenic bacteria strains Orial., (1992)

Heterotrophic bacteria derive their energy and carbon requirements from organic sources. Autotrophs are bacteria, which obtain their energy from light or by oxidation of inorganic materials and their carbon assimilation. Sulphur reducing bacteria, Sulphur oxidizing bacteria, Sulphide oxidizing bacteria, Iron oxidizing bacteria, Manganese oxidizing bacteria and Acid producing bacteria are the major microbes involved in microbial corrosion.

MATERIALS AND METHODS

Sample collection

The scale samples were collected from oil well at offshore platform (CAIRN energy), Rajamundari, India. The natural scale samples were collected and stored in sterile polythene bags. These samples were stored in an ice box and transported for microbiological characterization to Biocorrosion laboratory, CSIR-CECRI at Karaikudi, Tamilnadu

Isolation of calcium precipitating bacteria

The collected samples were in crystals form and the samples were grained with the help of mortar and pestle to bring those samples to powder form.Total viable bacterial counts were enumerated by pour plate technique using B4 medium for isolating calcium precipitating bacteria. The bacterial counts were made after 2-3 weeks incubation. Petri plates with 30-300 colonies were selected and the total viable counts/gm was made based on the following formula.

$$\text{CFU} = \frac{\text{Average number of colony (av) X 10ml}}{\text{Dilution X volumes of sample added X weight of scale sample}}$$

(av) = average for triplicate samples

10ml = volume of diluents.

Bacterial identification and characterization

Morphologically dissimilar colonies were selected and isolated from B4 medium for calcium precipitating bacteria. The isolated colonies were purified using appropriate medium by streaking methods. The pure cultures were maintained in specific slats at 4ºC to keep the microbial strain viable. The genus identification was carried out using standards characteristics described in Bergey's manual of systematic bacteriolgy (vol IV)(Holt *etal*., 1994).

Cell morphology and characterization

Cell morphology was studied through Gram staining, motility test, endospore staining and Epi-fluorescence microscope. The isolates were identified with the help of morphological studies and biochemical characterization.

Metabolic activities

Starch hydrolysis

The starch medium was prepared, sterilized and then poured in to sterile Petri plates. The test culture was streaked on the medium and incubated at 37ºC for 24 hours. After incubation, 2 or 3 iodine crystals were placed in the petridish and the plates were warmed slightly. Iodine stars vaporizing and reacts with starch to produce blue colour. Clear zone outside the area of growth indicates the extent of starch hydrolysis.

Characteristics of marine thermophilic calcium precipitating bacteria

An IR spectrum represents a fingerprint of a sample with absorption peaks which correspond to the frequencies of vibrations between the bonds of the atoms making up the material. These samples were characterized by employing the FITR spectrum taken in the mid IR region of 400-4000$cm^{-1.}$ The spectrum was recorded using ATR (attenuated total reflectance) technique. The sample was directly placed in the KBr powder and the spectrum was recorded in the transmittance mode.

X-ray diffraction analysis

The nature of oil well scale and the biogenic scale was lab induce scale formation by calcium precipitating bacteria were analyzed XRD. The XRD model: X'pert PRO PAN, X-ray diffractometer with Syn master 793 software to identify the scale. The XRD pattern was recorded using computer XRD-system, JEOL and Model: JPX-8030 with CuKradiation (Ni filtered = 13418 Ao) at the range of 40 kv, 20A. the 'peak search' and 'search match' program built in software (Syn master 7935) was used to identify the peak table and ultimately for the identification of XRD peak.

Scanning electron microscope analysis (SEM).

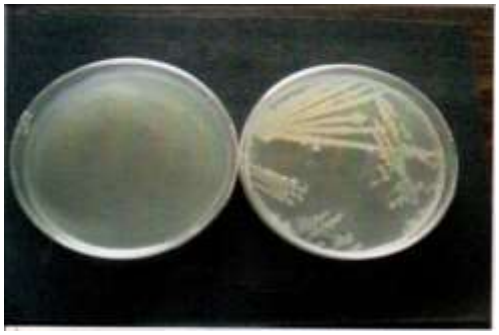
The scanning electron microscope consists of an energetically well focused beam of electrons across a sample. The microscope uses a LaB 6 source and is pumped using turbo and ion pumps to maintain the highest possible caccum. Secondary electron imaging (SEI) works on the principle that this electron beams generates a "splash" of electrons with kinetics energies much lower than the primary incident electrons called secondary electrons as a function of primary beam position makes it possible to attain high magnifications as much as x 100000 in some and high resolution (upto 40A) resolution for imaging the areas of interest. The coupons were observed by employing SEM (Hitachi model S-3000H). The coupons of the elements was analyzed by EDAX.

Results

Isolation of marine calcium precipitating bacteria:

The marine calcium precipitating was isolated from oil well scale collected from Rajamundri. The total viable bacterial count was $2.8x10^4$ CFU/gm. The morphologically different isolates were obtained in the marine scale sample. These cultures were used for further study. All these isolates were characterized by biochemical tests. The dominating isolates like CPB-1, 2 are positive in urease, catalase and MRVP tests. The selected isolates were grown at 50ºC temperature (Plate 1).

Plate 1: Isolation of calcium precipitating bacteria from scales on oil well platform in B4 medium



pH analysis

The initial pH of the B4 medium was 6.03. In contro system, the pH of the B4 medium was 5.59 at the end of 10^{th} day. In the presence of bacteria, pH was 6.02 slightly decreased similar to the control system and gradually increased with time 7.91. It clearly indicates that the bacterium is alkaliphilic organisms.

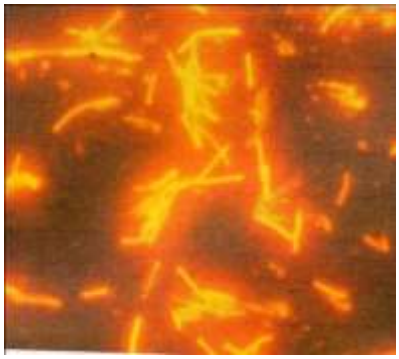
Bacterial growth rate based on temperature at 50ºC:

The initial growth of the bacteria in B4 medium at 50ºC was 0.1. In the control system, the growth of the B4 medium was 0.3 at the end of 10^{th} day. In the presence of bacteria, the growth was 2.5 gradually increasing. It indicates that bacterial growth at 50ºC temperature. It is cleary indicates that the bacterium is thermophilic bacteria.

Acridine orange staining:

The Epi-fluorescence photographs while using acridine orange for marine thermophilic bacteria. The rod shaped calcium precipitating bacteria was observed on paint coated mild steel. It clearly indicates that a bacterium is attachment on the surface (Plate 2).

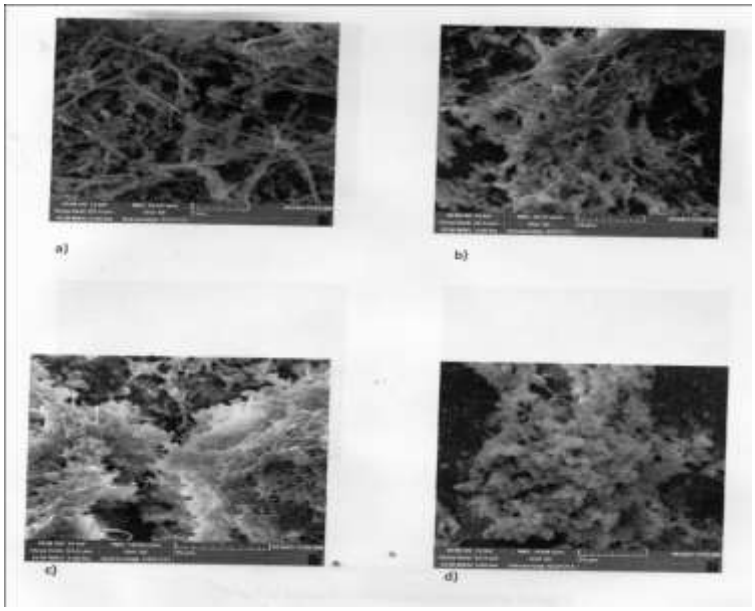
Plate 2: Epi-fluroscence microscope studies for scales collected from B4 medium in the presence of calcium precipitating bacteria



SEM analysis:

The morphology of cultured marine thermophilic bacteria presented in (plate8). The size of crystals was noticed approximately nm in presences of bacteria. These are generally either aggregate of planner, spherical, rod crystals shaped. It can be seen that bacterial cells are attached with crystal surfaces. This study confirms that the crystal formation occurs due to marine thermophilic bacteria. The temperature is one of the causative factors for the aggregation of biogenic crystals in presence of marine thermophilic bacteria on the paint coated mild steel. Two paint coating materials were visualized (Black coupons, and Brown coupons). In Brown coupons bacterial attachment in large amount (Plate 3).

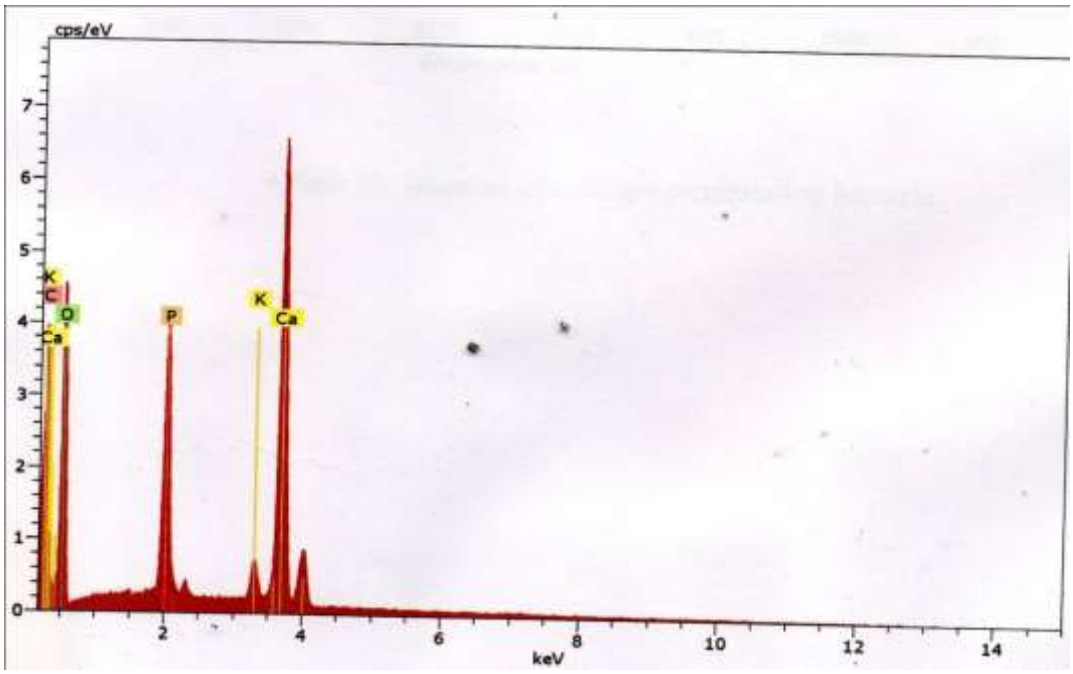
Plate 3: SEM studies for calcium crystals formation collected from B4 medium in the presence of calcium precipitating bacteria



EDAX:

Calcium element level has the higher in the calcium precipitating bacteria, when compare to other spots. The EDAX reveals that calcium precipitating bacteria contains micro crystals accumulation on the cell wall (Fig 1).

Fig 1: EDAX studies for calcium crystals formation collected from B4 medium in the presence of calcium precipitating bacteria

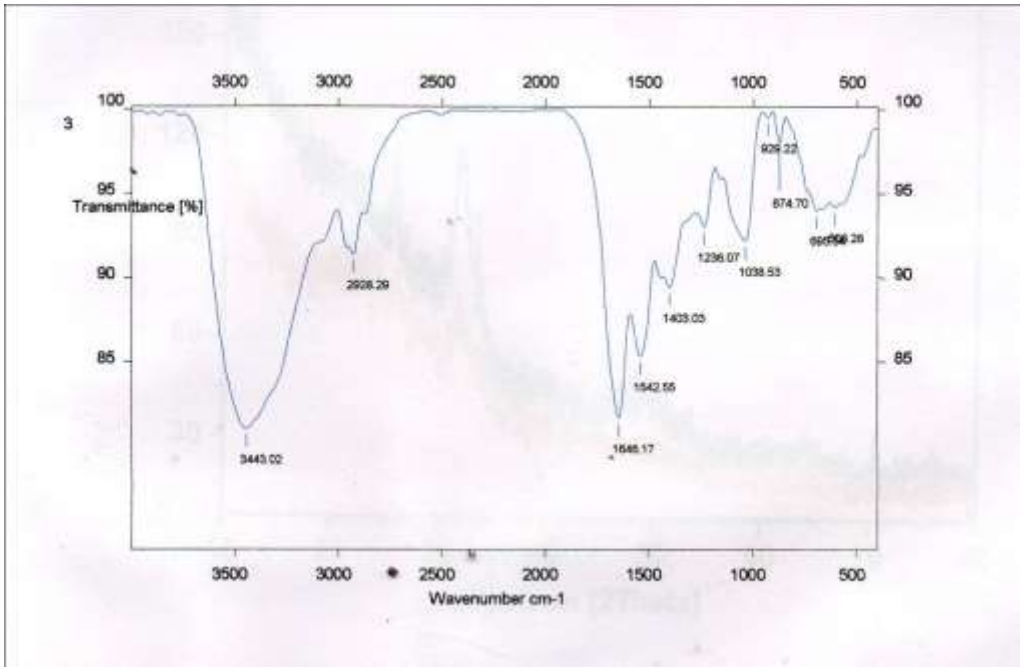


FTIR analysis:

Oil well peak noticed at 3434.06cm^{-1} can be assigned to the O-H stretching vibration of aromatic and alkyne's compounds. The corresponding bending vibrations were noticed at 2923.91 cm^{-1} can be assigned to the C-H stretching vibration of aromatic and alkyne's compounds respectively. A peak observed at can be assighned to the P-H stretching vibration. The peak observed 1642.26 cm^{-1} can be assigned to the C=O stretching vibration of metallic carbonates. Upon calcium ion binding with the COO^- groups the carbonul band at 1549.05 cm^{-1} the two peaks were observed at 137079 cm can be assigned to the P-O-H bands.The scale noticed at 3443 cm^{-1} can be assigned to the stretching vibration of primary and secondary amines. The corresponding bending vibrations were seen at 2928 cm^{-1} can be assigned to the C-H stretching vibration of alkane compounds respectively. A peak observed at 2517.71 cm^{-1} can be assigned to the P-H stretching vibration. The peak observed 1646.17 cm^{-1} can be assigned to the $(CO4)^2$ stretching vibration of metallic carbonates. Upon calcium ion binding with the COO^- groups the carbonyl band at 1542.05 cm^{-1}. The peaks were observed at 1038.03 cm^{-1} can be assigned to

the P-O-H bands. The two peaks observed at 1403.03 cm^{-1} can be assigned to the Caco3 stretching vibration of metallic carbonates. Another peak observed at 874.70 cm^{-1} that provide evidence of the formation of mixed phase of calcite and aragonite. The peak noticed 695.54 and 606.28 cm^{-1} can be assigned to the PO_4^{2-} stretching vibration. FTIR reveals the increased adsorption of calcium carbonite accumulation. The FTIR study reveals that the organic functional groups of carboxylic, secondary amines, hydroxyl and phosphate groups are corresponding to the formation of calcium precipitation on the bacterial biofilm. The biogenic functional groups can act as nucleating site for calcium mineralization and scale formation(Fig 2).

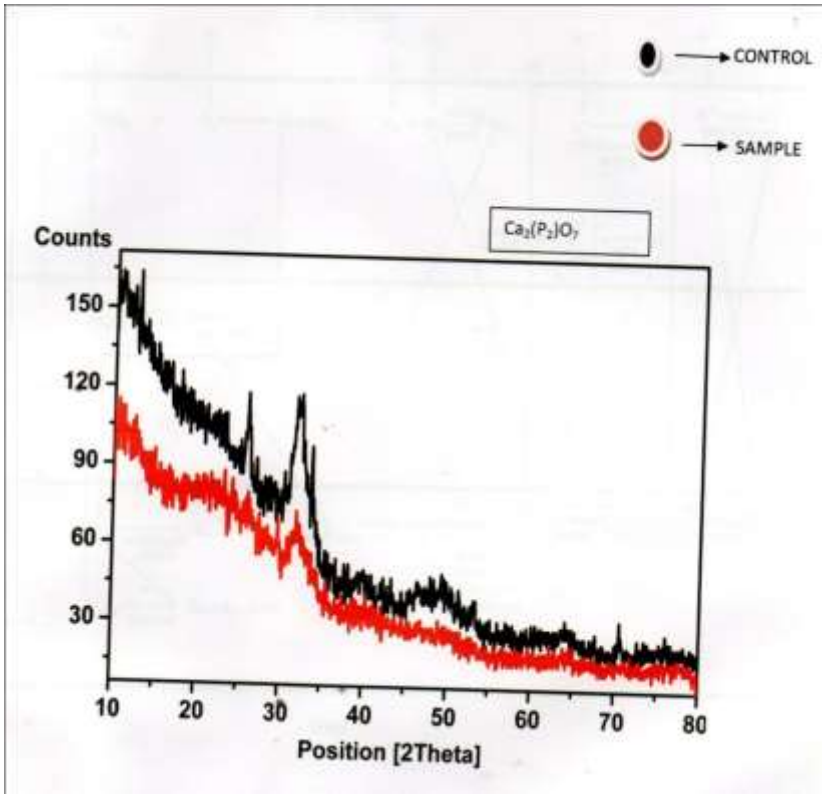
Fig 2: FTIR analysis for presence of calcium precipitating bacteria



X-Ray diffraction analysis:

Fig 5 represents that the results of XRD patter scales from oil well. This result indicates presence of $CaCO_3$ with high intensity was noticed. Calcium phosphate oxides are such as Ca_3P2O, $Ca_2P_2O_7$, Ca_4P_2O, Ca3 $(PO_4)_2$, Ca_{10} $(PO_4)6O_2$, also noticed. it indicates the presence of metal oxides and carbonates are present in the oil well scale at offshore platform. The crystals phase of $Ca_2(P_2O_7)$ peaks were high intensity when compared to control system (with out bacteria). The intensity of $Ca_2P_2O_7$ crystals phase peak depends upon the biological activities and dormation of calcium crystals (Fig 3).

Fig 3: XRD analysis of scales collected from oil well at offshore platform and calcium precipitating bacteria cultured in laboratory



DISCUSSION

Microbial associations with carbonate deposits have been described for seawater (Braissant *et al*., 2002) Saline lakes (Knorre *et al*.,2000) and soils (Morita, 1980). The occurrence of human kidney stone development (Boquet *et al*., 1973) as well as the deposits on the Martian meteorite (Kramer *et al*., 2000) and the phenomenon of nano forms were hypothesized to be in association with microbial classification (McKay *et al*., 1996). But no study has been carried out on calcium precipitation by bacteria at high temperatures. In this study, the occurrence of crystal formation in marine water system has been reported. The identified isolates showed positive for urease activity. The results supports the observation made by previous investigators (Fujita *et al*., 2000 and Vali *et al*., 2001), who noticed urease positive in CPB. Hammes and Verstraete (2002) also noticed that microbial carbonate precipitation by *Synechococcus Nannochlorisatomus*, *Bacillus* spp., *Pseudomonas* spp., *vibrio* spp., *flavobactrium* spp., and *Acinetobacter* spp. Photosynthetic- induced calcification is regarded as the most common form of microbial calcium precipitate (MCP), and is associated with algae or cynobacteria in primarily aqueous environments such as marine and/or fresh water (McConnaughey *et al*., 1997).

The calcium – accumulating process is based on metabolic utilization of organic acids as their role as carbon and energy. Such acids include acetate, citrate, oxalate, glyoxylate, succinate and malate and consumption of these acids results in a pH increase for the system, and thereby leading to precipitation in the presence of calcium ions (Fujita *et al*., 2000 and Vali *et al*., 2001) . in this study, it is possible that the identified species may consume calcium acetate and produce organic acids which results in a reduction of pH. The low pH shifted to higher side as per the following reaction. In the presence of bacterial system, there is no significant gradual reduction of the pH. At the end of the 10^{th} day, the pH was as high as 7.91 it clearly indicates that the presence of calcium acetate may reduce the pH initially which encourages the formation of free calcium ions. The calcium ions will be accumulated by bacteria as calcium carbonate and calcium phosphate crystals by the cell wall due to high pH which was identified by XRD. At the end of the 10 days of the experiment, the pH in all the cultures had reached about 7.91. the growth of the bacteria reaches maximum at 10^{th} day (2.5) in the presence of 50ºC. it may be due to the OH^- production due to bacterialactivity in calcium acetate. In the presence of bacteria, the formations of CO_2 and OH^- ions determine the pH of the solution. The continuous formation of OH enhances the pH and probability of calcium accumulation on the metal surface.

In this study, CPB collected from the sea water environment was observed. The growth of calcium carbonite crystal by various sizes and shapes dpend on the environmental condition. The crystals were identified by employing optical microscope. In he presence study the crystal formations by marine thermophilic bacteria were observed from 50ºC. stocks-Fischer *et al.,*(1999) have examined the physical and biochemical properties of $CaCO_3$precipitation in sand and found that only rhombohedral calcite crystals acting as nucleation sites. SEM observation (Plate 5&6) indicates the occurrence of both bacteria and calcite where the crystals provide substrate for the bacteria to develop colonies and the colonization either promotes or retards the further growth of the crystals faces (Lian *et al.,*2006 and tourney *et al.,2009*). Chen *et al.,(2009).* Also noticed that urease generated bacterium, *P. mirabilis,* is able to convert urea to ammonis and CO_2 and there by leading to the precipitation of metal carbonates. The present investigation claims that the presence of minor quantities of acetate may involve in the calcium crystal formation

CONCLUSION

On the basis of results, it is concluded that the calcium precipitating bacteria, one of the causative factor of crystals formation in oil well offshore platform. In the presence of hardness (Calcium and Magnesium), the utilized by bacteria at the acidic environment. The bacterial metabolic derivative of CO_2 reacts with calcium ions form $CaCO_3$ at the medium pH was increased.

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