

Characterization of Struvite Produced by an Algal Associated Agarolytic Bacterium *Exiguobacterium aestuarii* St. SR 101

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Abstract

Biomining phenomenon of bacteria proved to have various biotechnological and environmental applications. Production of magnesium ammonium phosphate (struvite) crystals by the agarolytic bacterium *Exiguobacterium aestuarii* St. SR 101 isolated from red seaweed, *Gracilaria corticata* was reported for the first time in the present study. Struvite crystallization occurred in the agar culture medium in the presence of the bacterium. Crystal nucleation and growth occurred apparently as a consequence of the localized ion supersaturation, produced by the microbial metabolites and also by the microbial supply of heterogeneous nuclei resulted in crystallization. The crystals were visible between 10 to 15 days after inoculation. The crystal structure of the struvite characterized by optical microscopy, IR spectroscopy, thermogravimetry, powder X-ray diffractometry, and single crystal X-ray diffractometry. The orthorhombic crystal is with the space group Pmn2₁, and unit-cell parameters a = 6.9447 Å, b = 6.1329 Å, c = 11.2026 Å. *Exiguobacterium aestuarii* St. SR 101 showed to have the capacity of producing struvite based fertilizer by bioremediation of industrial phosphate wastes.

Keywords: *Exiguobacterium aestuarii*; biomining; struvite; agarolytic; X-ray diffractometry.

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INTRODUCTION

Biom mineralization refers to a process which involves the formation of minerals. Bacterial activity related to the precipitation of a wide range of minerals including carbonates, oxides, phosphates, sulfides, and silicates¹. The extracellular precipitation processes of mineral compounds by bacteria are due to metabolic activity that favors the physicochemical conditions resulting in the formation of biominerals. In natural biomineralization, bacterial metabolic enzymes can generate supersaturated conditions. Moreover, it is an established and experimentally proven fact that bacterial structures participate in the extra or intracellular accumulation of inorganic material^{2,3}. The significant role of bacterial biomineralization is to deliver structures that serve as substrates for heterogeneous crystal nucleation (with lower energy barriers than homogeneous nucleation) and stereochemical arrangements of the mineral components. Therefore, providing the necessary elements and conditions and the mere existence of bacteria would themselves suffice for biomineralization processes^{4,5}. Interaction between soil bacteria and minerals reported having many bio-technological and environmental applications^{6,7}.

Among phosphate containing biominerals, struvite, $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$, has attracted considerable attention because of its common occurrence in widely diverse environments, such as bat droppings, decomposed foods, a part of biofilms that develop inside the pipelines in water treatment facilities. Crystallization of nitrogen and phosphorous in the form of struvite has been successfully used for nutrient removal and for obtaining a valuable fertilizer. The mineral struvite was named already in 1845 after the Russian Mineralogist, Heinrich Von Struve. According to Robinson's proposals, struvite production could be the consequence of a combination of ammonium ions formed by the metabolism of nitrogenous compounds with phosphate and magnesium present in the environment⁸. Several soil-inhabiting genera like *Azotobacter*, *Bacillus*, *Myxococcus*, and *Pseudomonas* have been studied for their potential to form struvite². Medical reports reveal that struvite is well known from the bladder and urinary concretions.

Due to its importance in many fields

of interest, it was thoroughly investigated by mineralogists, chemists, physicians, and environmentalists, who studied it from different points of view and described many physical and chemical properties. The crystal structure of struvite by Whitaker and Jeffery belongs to the orthorhombic space group $\text{Pmn}2_1$ with two molecules in a unit cell⁹. There are many structural similarities between struvite type materials, which have been of great interest because of their broad and important biological, agricultural and industrial implications^{10,11,12}. For example, Mg^{2+} cations in these compounds are all coordinated octahedrally by six H_2O molecules whose H atoms are strongly bonded to oxygen atoms in $(\text{PO}_4)^{3-}$ groups. No H_2O molecules are shared between $\text{Mg}(\text{H}_2\text{O})_6$ octahedra. Another common feature among the struvite type phosphate compounds is the face-sharing between PO_4 tetrahedra and $\text{Mg}(\text{H}_2\text{O})_6$ octahedra through hydrogen bonding, although the number of shared faces may vary from structure to structure. The present study reported a phosphate containing, biologically formed crystal, struvite, which has the chemical composition $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$, from the agarolytic bacterium *E. aestuarii* St. SR 101 isolated from the red seaweed, *G. corticata*.

MATERIALS AND METHODS

Isolation and identification of the bacterium

The agarolytic bacterium *E. aestuarii* St. SR 101, isolated from red seaweed *G. corticata* collected from Kovalam coast, near Chennai, India. The homogenized algal sample inoculated in medium containing 1.5% agar, 0.05% yeast extract, 0.5% peptone, 3% NaCl, 0.06% NaH_2PO_4 , and 0.01% CaCl_2 at room temperature (24 - 27°C) and under static condition. Within 3 days, the agar medium became liquefied due to the production of agarolytic enzyme agarase. After 10 to 15 days crystals were present in the medium. The crystals were harvested by washing thoroughly using double distilled water, air dried and stored in glass vials. The isolated bacterial stain characterized as described previously^{6,13}.

Effect of four different nutrients on the production of struvite

Four different ingredients of the basal medium viz., Peptone, yeast extract, NaH_2PO_4 and MgSO_4 were amended at seven different

concentrations (Peptone- 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1%; Yeast extract - 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1%; NaH_2PO_4 - 0.05, 0.06, 0.07, 0.08, 0.09, 0.1% and MgSO_4 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1%), and the bacterium was inoculated separately under respective concentrations in the medium without CaCl_2 , NaH_2PO_4 and MgSO_4 . The flasks were periodically observed for the presence of crystals, under room temperature at static condition. The crystals were collected after 15 days and transferred to distilled water and washed free of impurities. They were air dried, and the dry weight was recorded. Uninoculated culture medium was considered as negative control.

Characterization of crystals

The crystals obtained from the culture medium were ground into a fine powder and analyzed under powder X-ray diffraction using an X'pert PRO (PAN analytical) diffractometer with nickel-filtered CuK radiation in the 2θ range 5-80° with scan step size 0.02°/s. Fourier transform infrared of the crystals was recorded in the range of 400-4000 cm^{-1} using Perkin – Elmer RX1 FT-IR spectrophotometer in the form of solids dispersed in KBr pellets. Thermal analysis was carried out using SDT Q600 V8.0 Build 95 between 0°C and 900°C by thermal gravimetric analysis. Based on the optical examination, a prismatic crystal of 0.3 x 0.2 x 0.2 mm was selected and mounted on a CAD-4 Enraf-Nonius single crystal X-ray diffractometer equipped with graphite mono-chromatized $\text{MoK}\alpha$ radiation. A hemisphere of three dimensional X-ray diffraction data was collected with frame widths of 0.3°.

Table 1. Effect of different nutrients on the yield of crystals.

Peptone (%)	Yeast (%)	NaH_2PO_4 (%)	MgSO_4 (%)	Yield (mg)/100 L
0.4	0.04	0.05	0.4	16.0
0.5 (C)	0.05 (C)	0.06 (C)	0.5 (C)	30.9 (C)
0.6	0.06	0.07	0.6	54.5
0.7	0.07	0.08	0.7	56.5
0.8	0.08	0.09	0.8	97.0
0.9	0.09	0.10	0.9	125.9
1.0	0.10	0.20	1.0	144.9

(C) – Positive Control

RESULTS AND DISCUSSION

Culture conditions of *E. aestuarii*

The bacterium *E. aestuarii* St. SR 101 grown in different cultural conditions concerning the yield of struvite revealed the following observations. The amount of struvite produced by the bacterium was directly proportional to the concentration of the ingredients tested. Increasing concentrations of the combination of peptone, yeast extract, NaH_2PO_4 , and MgSO_4 increased the yield of struvite under the static condition at room temperature. The organism grown in the medium contained 0.1% yeast extract, 1.0% peptone, 3.0% NaCl , 0.2% NaH_2PO_4 , and 1.0% MgSO_4 showed a maximum of 145.0 mg/100 mL, which was 5 fold more than positive control (30 mg/100 mL) (Table 1). In the present study, no precipitation occurred in the negative control. These results are similar to those observed on moderately halophilic bacteria^{14,15}.

The present bacterium did not produce struvite when the medium devoid of any one of the ingredients viz., CaCl_2 , NaH_2PO_4 and MgSO_4 . Thus, these inorganic chemicals showed great influence on mineral precipitation⁵. The metabolic activity of bacteria made a change in pH, ionic strength and ionic makeup of the media which modify their mineralogical tendency and also affect cell surface charges which in turn modify the proportions of Ca^{2+} and Mg^{2+} captured by the cell surface^{16,17}. According to Rivadeneyra et al. struvite formation by bacteria involves the adsorption of Mg^{2+} and PO_4^{3-} ions, which in conjugation with the release of NH_4^+ ions may form microenvironments suitable for precipitation of this mineral¹⁸. In the present study, crystal formation occurs in the medium supplemented with CaCl_2 , NaH_2PO_4 , and MgSO_4 . Phosphate and magnesium are the major ingredients, whereas ammonium is believed to be released from the cells during incubation. The medium supplemented with organic nitrogen sources like peptone and yeast extract help the metabolic formation of ammonium. The light, alkaline pH of the medium 7.5, favors the conditions for biomineralization for the crystallization of struvite. Micrographic crystalline structure struvite produced by *E. aestuarii* illustrated in Fig. 1.

Solubility

The struvite crystals were soluble up to



Fig. 1. Optical micrograph is showing the crystal structure of struvite obtained from *E. aestuarii* St. SR 101.

pH 5.4, and a further increase in pH decreased the solubility. The crystals were not soluble in neutral and alkaline pH. Struvite solubility was determined chiefly by the concentration of magnesium, ammonium and phosphate and pH of the solutions¹⁹. In the present study struvite crystals formed from *E. aestuarii* were soluble in acidic pH, and as the pH increased the solubility decreased. It was soluble in 0.1 M citrate buffer pH from 3.0 to 5.4.

Thermal Analysis

Thermogravimetric analysis of the crystals was carried out between room temperature and 1000°C in the nitrogen atmosphere. The thermogram obtained is presented in Fig. 2. The thermal decomposition of struvite formed from

E. aestuarii started at 60°C and ended at 180°C with the peak temperature at 121.47°C resulted in a weight loss of 51.02% which corresponded to six molecules of water and one molecule of ammonia²⁰. The resulting MgPO₄ seems to be thermally stable up to 900°C. Since the loss of water occurs at a specific temperature, they are presumed to escape from the crystal lattice rather than from the crystal surface. Powder X-ray diffraction pattern of struvite crystals (Fig. 3) has given the evidence of elements viz., Mg, P, N, O and H. The lattice parameters were evaluated and found to be a = 6.955 Å, b = 6.142 Å and c = 11.218 Å, which are in conformity of the single crystal values. The powdered XRD pattern of struvite crystals in the present study matched very well with that of the published pattern for struvite (JCPDS file No. 77-2303).

IR Spectroscopic analysis

Water molecules possess three modes of structural vibrations ν_1 , ν_2 , ν_3 . All these modes are infrared active and occur at 3652(ν_1), 1595(ν_2) and 3765 (ν_3) cm⁻¹ in the vapor phase. They show shifts in liquid and solid phases. In general, their positions in the liquid phase are at 3219, 1645 and 3405 cm⁻¹ where as in solid phase they are 3200, 1640 and 3400 cm⁻¹ where as aacm⁻¹ whereas in solid phase

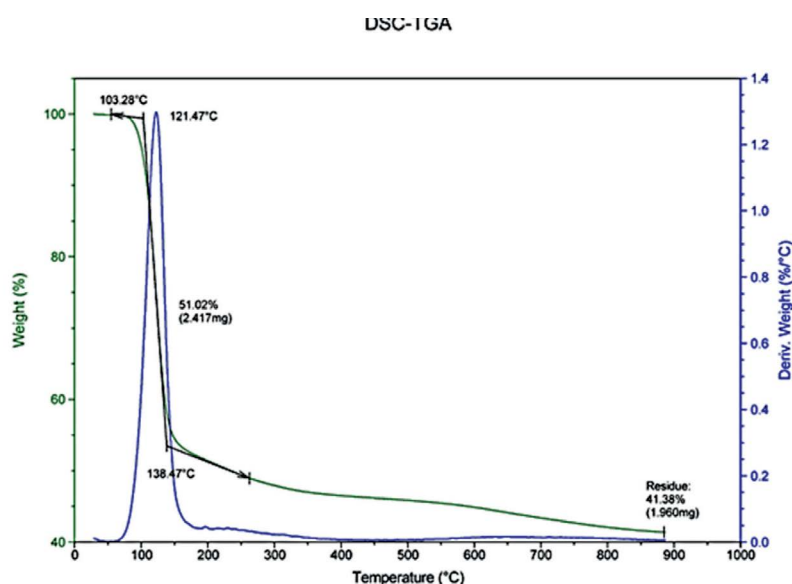


Fig. 2 Thermography of struvite crystals in the nitrogen atmosphere, showing a thermogravimetric peak at 121.47°C.

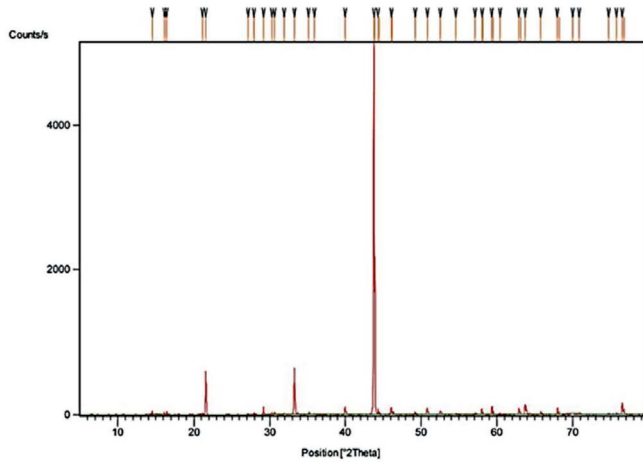


Fig. 3. X-ray diffraction powder pattern of struvite crystal.

they are at 3200, 1640 and 3400 cm^{-1} respectively. Such shifts are characteristics of hydrogen bonding in liquid and solid phases. The IR spectrum of the struvite obtained from *E. aestuarii* exhibits three bands characteristics of a water molecule at 1659, 3204 and 2924 cm^{-1} (Fig. 4). They are attributed to the structural vibrations ν_2 (bending mode), ν_1 (Symmetric stretch) and ν_3 (asymmetric stretch) respectively. The band of the struvite obtained from *E. aestuarii* culture medium observed at 750 cm^{-1} is assigned to water-water H-bonding²¹. The bands appeared at 457, 573 and 1005 cm^{-1} assigned to different modes of vibrations of PO_4 . The band at 1447 attributed to NH_3 . Therefore, the presence of PO_4 , NH_3 , and water in the struvite was confirmed.

Single Crystal X-ray Diffraction studies

Struvite crystal of size 0.3 x 0.2 x 0.2 mm was taken for data collection. The intensity data were collected using SMART CCD diffractometer ($\lambda = 0.7103$) at the Indian Institute of Technology, Chennai. The data collection was concealed over a hemisphere of reciprocal space by a combination of three sets of exposures and each set had a different θ angle (0° , 88° , and 180°) for the crystal and each exposure of 30 seconds covered 0.3° in ω . The crystal to detector distance was 4 cm, and the detector swing angle was -35° . Crystal decay was monitored by repeating 30 frames at the end of data collection (Table 2).

The intensities were collected with θ -range from 3.32 to 28.28° at 293 K. The values of

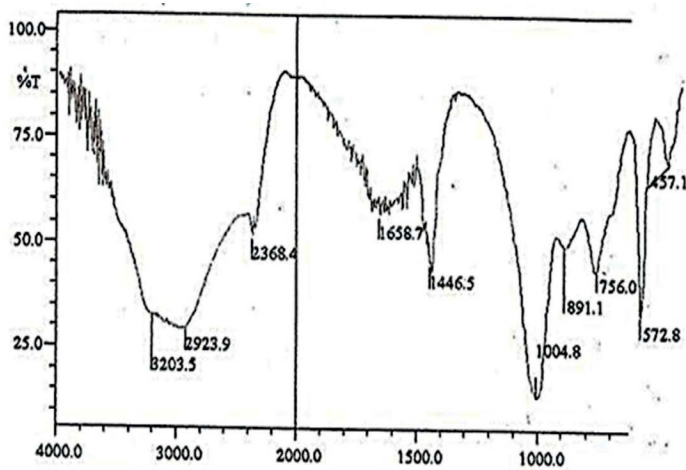


Fig. 4. IR spectrum of struvite crystal.

Table 2. Crystal data and structure refinement.

Empirical formula	H4 Mg0.25 N0.25 O2.50 P0.25
Formula weight	61.35
Temperature	293(2) K
Wavelength	0.71073 Å
Crystal system, space group	Orthorhombic, Pmn21
Unit cell dimensions	a = 6.9447(4) Å alpha = 90 deg. b = 6.1329(4) Å beta = 90 deg. c = 11.2026(7) Å gamma = 90 deg.
Volume	477.13(5) Å ³
Z, Calculated density	8, 1.708 Mg/m ³
Absorption coefficient	0.390 mm ⁻¹
F (000)	260
Crystal size	0.3 x 0.2 x 0.2 mm
Crystal description	Prismatic
Crystal color	colorless
Theta range for data collection	3.32 to 28.28 deg.
Limiting indices	-9<=h<=9, -8<=k<=8, -14<=l<=14
Reflections collected / unique	6653 / 1280 [R(int) = 0.0241]
Completeness to theta = 28.28	99.9 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	1.0 and 0.9
Refinement method	Full-matrix least-squares on F ²
Data / restraints / parameters	1280 / 17 / 106
Goodness-of-fit on F ²	1.376
Final R factors [$I > 2\sigma(I)$]	$R_1 = 0.0331$, $wR_2 = 0.0898$
Final R factors (all data)	$R_1 = 0.0332$, $wR_2 = 0.0898$
Absolute structure parameter	0.19(16)
Largest diff. peak and hole	0.477 and -0.304 e.Å ⁻³

hkl were $h \rightarrow -9$ to $+9$, $k \rightarrow -8$ to $+8$ and $l \rightarrow -14$ to $+14$. A total of 6653 reflections were collected and averaged basis on the symmetry equivalents. Unique reflections of 1280 resulted with $R_{int} = 0.024$. The completeness resulted in a maximum value of 99.9% for the $\theta \rightarrow 28.28^\circ$. The structure of struvite was solved by direct methods and refined by full-matrix least-squares procedures to a final R-value of 3.31% and weighted R-value of 8.98%. The crystal structure was solved by using SHELXL97²². The perspective view of the molecule is shown in Fig. 5.

The detailed study on the bond lengths and bond angles of the molecule revealed that the Mg environment assumes the octahedral arrangement. The neutron diffraction data on the ammonium group revealed three possibilities, namely (i) ammonium group might be either rotating about a fixed axis, (ii) rotating about a randomly oriented axis or (iii) fixed with structure by at least two bonds.

In this study, the ammonium group is fixed by two hydrogen bonds as follows:

N1–O5: 3.024(3) Å; 157.7(12)°; N1–O2: 2.784(4) Å; 173(4)°

The bonding nature is in extended form. A slight electrostatic imbalance is noted around ammonium N atom, but the features around the atom fulfill the conditions. The packing of the molecules indicates the well-formed hydrogen bonding for the ammonium group and the O atoms associated with Mg. Also, N–H...O and O–H...O types of hydrogen bondings lead to the network in the unit cell.

Struvite crystallizes in the orthorhombic system with cell dimensions $a = 6.9447 \text{ \AA}$, $b = 6.1329 \text{ \AA}$, $c = 11.2026 \text{ \AA}$. The space group is $Pmn2_1$, and there are two molecules in the unit cell. The structure of struvite crystals consists of regular PO_4^{3-} tetrahedra, distorted $\text{Mg}_6\text{H}_2\text{O}$ octahedra, NH_4^+ groups, which are held together by hydrogen

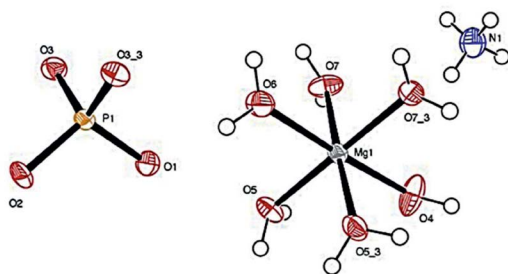


Fig. 5. Atomic coordination of struvite produced by *E. aestuarii* St. SR 101.

bonding. The structure of struvite crystals in the present study was in good agreement with that reported previously^{9,23}.

Yang and Sun suggested that phosphate metabolism or phosphate containing organic compounds could be a good source for inorganic phosphate²⁴. Ben Omar et al.²⁵ and Gonzalez-Munoz et al.²⁶ conducted a series of experiments on struvite formation with several soils inhabiting genera, like *Azotobacter*, *Bacillus*, *Myxococcus*, and *Pseudomonas*. In earlier research findings by Ben Omar et al. revealed that the struvite precipitation would not occur until the autolysis of *Myxococcus xanthus* cultures and the actual physical presence of the bacteria was necessary²⁷. Ben Omar et al. also found that dead cells, disrupted cells, and membrane fractions of this microorganism induce the struvite crystallization². All the reports lend support to this present biomineralization process of struvite.

In Environmental mineralogy, the precipitation of struvite from sewage has been a subject of considerable investigations because it may offer a potential route for dephosphorization of wastewater from industries and recover phosphates for recycling in the form of fertilizer. Struvite has many potential uses as a fertilizer. The most promising application is as a slow-release fertilizer that can be applied in a single high dose without damage to growing plants since this might have low leach rates and thus slowly releases nutrients in the given environment. At present, numerous research projects are focusing on the controlled precipitation of struvite and its later utilization as a fertilizer^{28,29,30}. A better understanding of its formation process, especially in terms of the degree of biological controlled

vs. biologically induced mineralization, will undoubtedly provide the additional knowledge in this regard and facilitate future work on biomineralization of struvite-type phosphate minerals^{31,32}.

The present study is the first report on biomineralization of struvite by an agarolytic bacterium since biomineralization occurs in viable biomass and this study elucidated the association of biomineralization with physio-logical traits of the bacterium which indicates the present struvite shall be a potentially useful Bioresource.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHORS' CONTRIBUTION

All authors have made substantial, direct and intellectual contribution to the work and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript and/or the Supplementary Files.

ETHICS STATEMENT

Ethical clearance was obtained from Institutional Ethical Committee

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